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Toxicological potential of semiconductor nanoparticles and their impact mechanisms to fish in early development

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Puslaidininkinių nanodalelių toksikologinis potencialas ir poveikio mechanizmai žuvims ankstyvajame jų vystymesi

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CONTENTS

ABBREVIATIONS

GVF – Gill ventilation frequency
HAB – Harmful cyanobacterial blooms
HR – Heart rate
MAA – Mercaptoacetic acid
MPA – Mercaptopropionic acid
MT – Metallothionein
NPs – Nanoparticles
OECD – Organization for Economic Co-operation and Development
PEG – Polyethylene glycol
PL – Photoluminescence
QDs – Quantum dots
RBMI – Relative body mass increase
ROS – Reactive oxygen species
TGA – Thioglycolic acid
UV – Ultraviolet

LIST OF PUBLICATIONS

The results of the present study are presented in 11 scientific publications (Papers I-XI) and were presented at 14 national and international scientific conferences. Publications are referred within the text using Roman numerals.

Publications with an impact factor on the Clarivate Analytics Web of Science database:

I. Rotomskis R, **Jurgelėnė Ž**, Stankevičius M, Stankevičiūtė M, Kazlauskienė N, Jokšas K, Montvydienė D, Kulvietis V, Karabanovas V (2018) Interaction of carboxylated CdSe/ZnS quantum dots with fish embryos: Towards understanding of nanoparticles toxicity. Science of the Total Environment 635: 1280–1291.

II. Cibulskaitė Ž*, Kazlauskienė N, Rotomskis R, Kulvietis V (2018) Toxicity of quantum dots and cadmium to rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) in early ontogenesis. Fresenius Environmental Bulletin 27(1): 241–245.

III. Jurgelėnė Ž, Kazlauskienė N, Montvydienė D, Kulvietis V, Rotomskis R, Jokšas K (2018) Embryotoxicity of Quantum Dots in Rainbow Trout *Oncorhynchus mykiss* During the Hatching Period. Bulletin of Environmental Contamination and Toxicology 101(2): 191–196.

IV. Šulčius S, Montvydienė D, Mazur-Marzec H, Kasperovičienė J, Rulevičius R, **Cibulskaitė Ž*** (2017) The profound effect of harmful cyanobacterial blooms: From food-web and management perspectives. Science of the Total Environment 609: 1443–1450.

Other peer-reviewed publications:

V. Cibulskaitė Ž*, Kazlauskienė N, Kulvietis V (2015) Sublethal toxicity of quantum dots and heavy metals to rainbow trout (*Oncorhynchus mykiss*) in early ontogenesis. Proceedings of the 18th Conference for Junior Researchers „Science – Future of Lithuania“, Environmental protection engineering. Vilnius, Lithuania, 31–37.

VI. Cibulskaitė Ž*, Stankevičiūtė M, Kazlauskienė N, Baršienė J, Kulvietis V, Rotomskis R (2016) Long-term toxicity and geno-cytotoxicity of quantum dots to rainbow trout *Oncorhynchus mykiss* embryos. Proceedings of the 13th International Conference on Protection and

Restoration of the Environment. Mykonos island, Greece, 460–470. ISBN: 978-960-6865-94-7.

VII. Kazlauskienė N, **Cibulskaitė Ž***, Stankevičiūtė M, Baršienė J. (2016) Experimental studies on the toxicity and geno-cytotoxicity effects of cadmium in embryos and larvae of rainbow trout, *Oncorhynchus mykiss*. Proceedings of the 13th International Conference on Protection and Restoration of the Environment. Mykonos island, Greece, 449–459. ISBN 978-960-6865-94-7.

VIII. **Cibulskaitė Ž***, Kazlauskienė N, Jokšas K, Kulvietis V, Makaras T, Stankevičius M, Rotomskis R (2017) Accumulation of Cd in the Early Stages of the Development of Rainbow Trout *Oncorhynchus mykiss* Exposed to Cd Based Quantum Dots and Cd Salt. 10th International Conference. Vilnius Gediminas Technical University, Vilnius, Lithuania, eISSN 2029-7092 / eISBN 978-609-476-044-0; doi.org/10.3846/enviro.2017.014.

IX. Montvydienė D, Makaras T, Kazlauskienė N, **Cibulskaitė Ž***, Šulčius S (2017) Ecotoxicity assessment of multicomponent mixtures of different origin (landfill leachate and biomass of harmful algae bloom) using three aquatic organisms. CEMEPE proceedings of 6th International Conference on Environmental Management, Engineering, Planning & Economics, Thessaloniki, Greece, 114–123. ISBN: 978-618-5271-15-2.

X. **Jurgelėnė Ž**, Stankevičiūtė M, Kazlauskienė N, Montvydienė D, Baršienė J, Jokšas K, Markuckas A (2018) Investigation of quantum dots toxicity, genotoxicity, cytotoxicity, and uptake in rainbow trout *Oncorhynchus mykiss* larvae. Proceedings of the 14th International Conference on Protection and Restoration of the Environment. Thessaloniki, Greece, 775–806. ISBN: 978-960-99922-4-4.

XI. Stankevičiūtė M, **Jurgelėnė Ž**, Greiciūnaitė J, Markovskaja S, Kazlauskienė N, Baršienė J (2018) Geno-, cytotoxicity and toxicity induced by *Saprolegnia parasitica* and cadmium alone and in combination to *Oncorhynchus mykiss*. Proceedings of the 14th International Conference on Protection and Restoration of the Environment Thessaloniki, Greece, 795–804. ISBN: 978-960-99922-4-4.

Presentations in national and international conferences:

1. **Cibulskaitė Ž***, Kazlauskienė N, Kulvietis V. Subletalus kvantinių taškų ir sunkiųjų metalų toksinis poveikis vaivorykštiniam upėtakiui (*Oncorhynchus mykiss*) ankstyvojoje ontogenezėje. Lietuvos jaunųjų mokslininkų konferencijos „Mokslas – Lietuvos ateitis“ antropogeninės

taršos poveikis aplinkai sekcijoje. 2015 m. balandžio 9 d. Vilnius, Lietuva. Oral presentation.

2. **Cibulskaitė Ž***, Kazlauskienė N, Rotomskis R, Kulvietis V. Toxicity of Quantum Dots and Cadmium to Rainbow Trout *(Oncorhynchus mykiss*) in early ontogenesis. XV European Congress of Ichthyology. The theme session "Physiology, Behaviour ad Toxicology". September 06-11, 2015, Porto, Portugal. Poster presentation.
3. **Cibulskaitė Ž***, Kazlauskienė N. Žuvis – biomedicininių tyrimų objektas. 19-oji Balt-LASA konferencija "Bandomieji gyvūnai moksliniuose tyrimuose". 2015 m. lapkričio 26 d. Vilnius, Lithuania. Oral presentation.
4. Kazlauskienė N, Rotomskis R, Kulvietis V, **Cibulskaitė Ž***. Embryotoxicity of Quantum Dots During Hatching Period in Rainbow Trout (*Oncorhynchus mykiss)*. XV European Congress of Ichthyology. The theme session "Physiology, Behaviour ad Toxicology". September 06-11, 2015, Porto, Portugal. Poster presentation.
5. Kazlauskienė N, **Cibulskaitė Ž***, Svecevičius G, Sauliutė G, Makaras T, Rotomskis R, Kulvietis V, Stankevičius M, Markuckas A, Stankevičiūtė M, Baršienė J. Nanoparticle And Heavy Metal Toxicity Mechanisms In Fish During Ontogenesis: An Interdisciplinary Project. The international Conference of Natural and Life Sciences The Coins 2016, February 29 - March 03, 2016, Vilnius, Lithuania. Poster presentation.
6. **Cibulskaitė Ž***, Stankevičiūtė M, Kazlauskienė N, Baršienė J. Toxicity and Geno-cytotoxicity of Cadmium to Rainbow Trout (*Oncorhynchus mykiss*) in early ontogenesis. International Conference, Vita Scientia, January 04, 2016, Vilnius, Lithuania. Poster presentation.
7. **Cibulskaitė Ž***, Stankevičiūtė M, Kazlauskienė N, Baršienė J, Kulvietis V, Rotomskis R. Long-term toxicity and geno-cytotoxicity of quantum dots to rainbow trout *Oncorhynchus mykiss* embryos. 13th International Conference on Protection and Restoration of the Environment, July 3-8, 2016, Mykonos island, Greece. Poster presentation.
8. Kazlauskienė N, **Cibulskaitė Ž***, Stankevičiūtė M, Baršienė J. Experimental studies on the toxicity and geno-cytotoxicity effects of cadmium in embryos and larvae of rainbow trout, *Oncorhynchus mykiss.* 13th International Conference on Protection and Restoration of the Environment, July 3-8, 2016, Mykonos island, Greece. Poster presentation.
9. Stankevičius M, **Cibulskaitė Ž***, Kazlauskienė N, Rotomskis R. Accumulation of Quantum dots in Rainbow Trout (*Oncorhynchus*

mykiss) Embryos. 59th Scientific Conference for Students of Physics and Natural Sciences "Open Readings 2016", March 15-18, 2016, Vilnius, Lithuania. Poster presentation.

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11. Stankevičius M, **Cibulskaitė Ž***, Kazlauskienė N, Rotomskis R. Fluorescence microscopy of quantum dots distribution in rainbow trout embryos chorion. 60th Scientific Conference for Students of Physics and Natural Sciences "Open Readings 2017", March 14-17, 2017, Vilnius, Lithuania. Poster presentation.
12. **Cibulskaitė Ž***, Kazlauskienė N, Jokšas K, Kulvietis V, Makaras T, Stankevičius M, Rotomskis R. Accumulation of Cd in the Early Stages of the Development of Rainbow Trout *Oncorhynchus mykiss* Exposed to Cd Based Quantum Dots and Cd Salt. "Environmental Engineering" 10th International Conference, April 27-28, 2017, Vilnius Gediminas Technical University, Vilnius, Lithuania. Poster presentation.
13. **Jurgelėnė Ž**, Stankevičiūtė M, Kazlauskienė N, Montvydienė D, Baršienė J, Jokšas K, Markuckas A. Investigation of quantum dots toxicity, genotoxicity, cytotoxicity, and uptake in rainbow trout *Oncorhynchus mykiss* larvae. Proceedings of the 14th International Conference on Protection and Restoration of the Environment. July 3-6, 2018, Thessaloniki, Greece. Oral presentation.
14. Stankevičiūtė M, **Jurgelėnė Ž**, Greiciūnaitė J, Markovskaja S, Kazlauskienė N, Baršienė J. Geno-, cytotoxicity and toxicity induced by *Saprolegnia parasitica* and cadmium alone and in combination to *Oncorhynchus mykiss*. Proceedings of the 14th International Conference on Protection and Restoration of the Environment. July 3-6, 2018, Thessaloniki, Greece. Oral presentation.

Conference abstracts:

I. **Cibulskaitė Ž***, Kazlauskienė N, Rotomskis R, Kulvietis V (2015) Toxicity of Quantum Dots and Cadmium to Rainbow Trout (*Oncorhynchus mykiss*) in early ontogenesis. Abstracts of XV European Congress of Ichthyology, 07 Sep 2015, Porto, Portugal. Front. Mar. Sci. doi: 10.3389/conf.FMARS.2015.03.00195.

II. Kazlauskienė N, Rotomskis R, Kulvietis V, **Cibulskaitė Ž*** (2015) Embryotoxicity of Quantum Dots in Rainbow Trout *Oncorhynchus mykiss* During Hatching Period. Abstracts of XV European Congress of Ichthyology, 07 Sep 2015, Porto, Portugal. Front. Mar. Sci. doi: 10.3389/conf.fmars.2015.03.00110.

III. Kazlauskienė N, **Cibulskaitė Ž***, Svecevičius G, Sauliutė G, Makaras T, Rotomskis R, Kulvietis V, Stankevičius M, Markuckas A, Stankevičiūtė M, Baršienė J (2016) Nanoparticle And Heavy Metal Toxicity Mechanisms In Fish During Ontogenesis: An Interdisciplinary Project. The international Conference of Natural and Life Sciences The Coins 2016, February 29 - March 03, Vilnius.

IV. Stankevičius M, **Cibulskaitė Ž***, Kazlauskienė N, Rotomskis R. Accumulation of Quantum dots in Rainbow Trout (*Oncorhynchus mykiss*) Embryos. 59th Scientific Conference for Students of Physics and Natural Sciences "Open Readings 2016", March 15-18, 2016, Vilnius, Lithuania. http://www.openreadings.eu/wp-content/uploads/2016/03/OR2016_abstract_book.pdf

V. Stankevičius M, **Cibulskaitė Ž***, Kazlauskienė N, Rotomskis R. Fluorescence microscopy of quantum dots distribution in rainbow trout embryos chorion. 60th Scientific Conference for Students of Physics and Natural Sciences "Open Readings 2017", March 14-17, 2017, Vilnius, Lithuania. http://www.openreadings.eu/wp-content/uploads/2017/03/OR2017_abstracts_book.pdf

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[1] Asterisk (*) indicate the change of surname of Živilė from Cibulskaitė to Jurgelėnė.

[2] Declaration of contribution

The study experiments were designed by Ž. Jurgelenė in cooperation with dr. N. Kazlauskienė and prof. habil. dr. R. Rotomskis. Ž. Jurgelėnė initiated and planned Papers I–III, V–VIII, X, was responsible for the analysis of test-parameters (Table 1), and statistic (Papers I, III, V–VIII, X). Ž. Jurgelėnė was responsible for all parts related to biological parameters assessment of fish and preparation of certain parts in Papers IV, IX, XI.

INTRODUCTION

The active development of nanotechnology has led to the emergence of a new class of environmental pollutants, i.e. nanoparticles (NPs), which (in particular, those containing metals) may significantly affect the environment and human health (Hardman 2006; Blickley et al., 2014; Rocha et al., 2017; Paper III). One type of Cd-based NPs are semiconductor quantum dots (QDs), which have a range of unique properties making them interesting for manifold photo-physical applications (Alivisatos et al., 1996; Gagne´ et al., 2010; Piccinno et al., 2012; Paper I).

QDs are colloidal nanostructured materials composed of a semiconductor core (e.g., CdSe, CdTe) (Paper I). CdSe is often coated with wider-bandgap materials (such as ZnS) that also act as protection of the core, prevent Cd leaching and enhance photoluminescence (Domingos et al., 2011; Zarco-Fernández et al., 2016; Paper VIII). QDs may have organic coatings that increase their dispersion in water and help to direct them to biological targets (Medintz et al., 2005; Paper VIII). QDs exhibit such exceptional optical properties as photostability, high photoluminescence quantum yield, size tunable emission spectrum, broad absorption spectra, flexible surface engineering, etc. (Hardman 2006). Due to these properties and their wide applicability, the global production of QDs is increasing from year to year (Piccinno et al., 2012). The main areas of QDs application are healthcare, quantum computing, quantum optics, optoelectronics, energy and security (Paper I). Additionally, the key products in the global QDs market include solar cells, medical devices, lasers, lighting, light-emitting diode displays and sensors (Piccinno et al., 2012). However, limited data are available on the risks posed by QDs to environmental health, where potential sources of toxicity might occur due to their uptake by organisms from different trophic levels (Rocha et al., 2017). The question about QDs safety is still open, because their toxicological effects have not been fully investigated.

1. Fish in early development as a nanotoxicity model

Due to their relatively short embryonic development duration, fish are widely used as a vertebrate model in biomedical research for a variety of purposes, including drug safety screening, elucidation of human disease mechanisms and environmental health assessment, etc. (Powers 1989; Yong et al., 2013; Rocha et al., 2017; Paper I). In recent years, the use of fish as an established animal model system in nanotoxicity studies has been growing

exponentially (Chakraborty et al., 2016; Rocha et al., 2017). Among the fish species most commonly used as models in nanotoxicity studies are the zebrafish (*Danio rerio*) and the rainbow trout (*Oncorhynchus mykiss*) (Rocha et al., 2017). These species are also widely used in standard toxicological testing of chemicals (ISO 12890:1999; ISO 7346-1:1996; ISO 10229:1994; Hrovat et al., 2009). However, due to its longer embryonic development, which allows conducting prolonged experiments, the rainbow trout is more advantageous as a model system than the aquarium fish (Ballard 1973; Vosylienė 2007; Paper I).

Different types of parameters are used to evaluate nanotoxicity: hatching achievement rate, developmental malformation of organs, lesions of gills and skin, abnormal behaviour (movement impairment), immunotoxicity, genotoxicity or gene expression, neurotoxicity, endocrine system disruption, reproduction toxicity and, finally, mortality of fish (Chakraborty et al., 2016; Paper X).

Embryonic development of fish is an important model for assessing toxicity as well as transportation of NPs in tissues (Lee at al., 2007; Kang et al., 2015). According to Murugan et al. (2015), NPs can enter biological membranes. For this reason, the interest in the chorion ability to protect the fish embryo from NPs until hatching is increasing (Kashiwada 2006; Lee et al., 2007; Asharani et al., 2008; Browning et al., 2009; Fent et al., 2010; Osborne et al., 2013; Kang et al., 2015).

2. QDs toxicity

Toxicity of QDs is a growing problem, especially due to their nano-specific properties, physico-chemical transformation in the environment and release of toxic metals from the QDs core (Ipe et al., 2005; Hardman 2006; Ribeiro et al., 2012; Katsumiti et al., 2014; Devin et al., 2016; Rocha et al., 2017; Paper I).

Toxic effects of QDs have been studied on different animal species, such as the nematode *Caenorhabditis elegans* (Qu et al., 2011), silkworm *Bombyx mori* (Liu et al., 2014), fresh-water polyp *Hydra vulgaris* (Ambrosone et al., 2012), zebrafish (Zhang et al., 2012a; 2012b), rainbow trout (Federici et al., 2007; Shahbazzadeh et al., 2009; Scown et al., 2010; Munari et al., 2014) and mice (Chu et al., 2010; Scoville et al., 2015; Wang et al., 2016).

QDs induce various dose- and age-dependent toxicity endpoints in zebrafish, including increased mortality, reduced growth, necrosis, yolk sac malformation and malformed tail (King-Heiden et al., 2009; Leigh et al.,

2012; Zhang et al., 2012b; Zolotarev et al., 2012; Duan et al., 2013; Rocha et al., 2017; Paper VI).

However, QDs toxicity to rainbow trout at early development stages has not been investigated. Further investigations are needed to clarify prolonged toxicity mechanisms of QDs in organisms, particularly in those at early development stages.

3. Toxic components in QDs

Toxicological studies have revealed that QDs toxicity to fish occurs when QDs degrade and metal ions leak out (Gagné et al., 2010; Paper II). The experiment findings reported by King-Heiden et al. (2009) showed that such chemical elements as Zn, Se and S, (except Cd) that are present in CdSe/ZnS QD do not cause significant toxicity to zebrafish at early development stages. King-Heiden et al. (2009) found that in some cases QDs toxicity was different from that of Cd. Therefore, it is necessary to examine different aspects of QDs toxicity when comparing it with that of Cd (Paper II).

Cd is a nonessential metallic trace element widely distributed in the aquatic environment (Annabi et al., 2013; Pereira et al., 2015; Paper VIII). Nanotechnology also poses risk to this metal (Rzigalinski and Strobl 2009). Several field studies demonstrated that Cd contamination could persist for many years in the aquatic environment because of its storage in sediments and its further release into the water column under favorable hydrodynamic conditions (Coynel et al., 2007; Paper VIII). This could be the reason for long-term Cd accumulation in aquatic organisms (Baudrimont et al., 2005; Paper VIII).

Cd toxicity to different freshwater fish species has been extensively investigated (Al-Asgah et al., 2015). Cd exerts a wide range of pathological effects on fish and other aquatic organisms (Al-Asgah et al., 2015). Some authors disclosed Cd treatment-induced morphological, physiological, hematological, biochemical and immunological changes in the test fish at early development stages (Brinkman et al., 2007; Ismail and Yusof 2011; Heydarnejad et al., 2013; Paper VII).

4. Accumulation, penetration and distribution of QDs in fish

Most studies focused on the embryonic toxicity of QDs rather than on the assessment of QDs accumulation, penetration and distribution in fish embryos and larvae (Zhang et al., 2012b; Zolotarev et al., 2012; Duan et al.,

2013; Rocha et al., 2017; Paper VIII). Data about Cd concentration levels in fish at early life stages and possible Cd leakage from QDs structures are scarce. King-Heiden et al. (2009) demonstrated that metallothione (MT) expression could be used as a marker of internal Cd exposure, providing indirect information on *in vivo* QDs degradation. The study by Zarco-Fernández et al. (2016) demonstrated that Cd from Cd salts and from CdSe/ZnS QDs accumulates in different areas of zebrafish larvae.

Cd accumulation in organisms depends on the concentration, route of uptake and environmental conditions (Karakoç and Dinçer 2003; Bowen et al., 2006; Jezierska and Witeska 2006; Guinot et al., 2012). Meanwhile, the accumulation of QDs is predetermined by its composition, size and surface chemistry (Zarco-Fernández et al., 2016; Paper VIII).

The information on QDs uptake and distribution in fish is relatively sparse when compared to the knowledge of other NPs (Kashiwada 2006; Lee et al., 2007; Asharani et al., 2008; Browning et al., 2009; Fent et al., 2010; Osborne et al., 2013; Böhme et al., 2015; Kang et al., 2015; Paper I). Behaviour of various NPs in zebrafish embryos has been studied (Lee et al., 2007; Asharani et al., 2008; Browning et al., 2009; Fent et al., 2010; Zolotarev et al., 2012; Böhme et al., 2015). However, there is no information regarding QDs accumulation, penetration and distribution in rainbow trout embryos (Paper I). According to Rocha et al. (2017), the possible effects of QDs on fish embryonic development are related with the protective properties of chorion. However, there is no detailed information on penetration abilities of QDs through the chorion of embryos and larval body tissues. Only Zolotarev et al. (2012) and Petushkova et al. (2015) noticed the formation of large structures of QDs particles on the chorion surface of zebrafish embryos. However, they did not mention and investigate the aggregation of QDs. Meanwhile, King-Heiden et al. (2009) noted that during the aggregation process in test-water, hydrodynamic diameters of QDs increased. Furthermore, there are no spectroscopy and microscopy data about the visualization of QDs accumulation in a fertilized egg and interaction of QDs with the chorion of embryos (Paper I). Although, confocal microscopy is widely used in biomedical research (Bijeesh et al., 2017), its employment for the QDs impact assessment on living organism and their 3D reconstruction images is still poor (Paper I). No data are available on the study of QDs aggregation in the chorion of living embryos, because most visualization methods require animal death (Chen et al., 2011; Brun et al., 2014; Lee and An 2014; Böhme et al., 2017; Paper I).

5. Mechanisms of QDs impact on fish

The question about QDs impact mechanisms is still open: metal toxicity or specific nanotoxicity of QDs has an influence on the early stage of fish embryogenesis (Ipe et al., 2005; Ribeiro et al., 2012; Katsumiti et al., 2014; Rocha et al., 2015; Santana et al., 2015; Paper I).

According to Blickley et al. (2014), oxidative stress could be the main mechanism by which QDs induced toxicity. QDs and their degradation products generate ROS, imbalance of pro- and anti-oxidant processes (Basha and Rani 2003; Blickley et al., 2014). On the other hand, the understanding of the stable QDs' ability to pass biological barriers and to induce negative effects is still limited. Therefore, the impact of QDs could be explained by studying the effects of other NPs on aquatic organisms. The possible molecular mechanism of Ag NPs toxicity was revealed by Gao (2016). At the embryonic stage, the fish chorion was covered with NPs, which blocked oxygen intake and caused bradycardia (Gao 2016). Hypoxia induced expression of vascular endothelia growth factor signalling pathway genes; at the same time, intracellular Ag NPs entered the endoplasmic reticulum and blocked protein synthesis. Afterwards, hypoxia blocked angiogenesis and caused mortality of zebrafish at a later development stage (Gao 2016).

Environmental organic (cyanobacterial blooms biomass (HAB)) or inorganic (clay) materials could also induce hypoxia, mechanically block chorion pores of fish embryos and adhere to the gills of adult fish and larvae (Lapointe et al., 2004; Shang and Wu 2004; Grieg et al., 2005; Julien and Bergeron 2006; Shang et al., 2006; Wyatt et al., 2010; Gao 2016). Eutrophication is a factor influencing embryo mortality in water bodies, and survival is the lowest when eggs are in contact with fine, muddy sediments (Wyatt et al., 2010). Fine materials on nano- and micro-scales have been shown to be detrimental to survival of salmonid species because they reduce oxygen delivery to embryos (Grieg et al., 2005; Louhi et al., 2008; Wu and Zhou 2012; Gao 2016). Therefore, it is necessary to investigate and compare the toxicological potential of engineered (QDs) and environmental (HAB and clay) nano-scale materials to aquatic organisms.

Consequently, research on the prolonged effects of QDs designed to gain a better understanding of their impact on fish, other aquatic organisms and humans is important and needs to be continued. Toxicological studies of QDs can provide data on their acute and chronic toxicity to aquatic organisms, relationships between nano- and micro-scale materials and aquatic organisms, and, thus, reveal impact mechanisms of other NPs and

provide a foundation for the knowledge-based management of aquatic ecosystems.

6. Scientific novelty of the thesis

1. For the first time, the toxicological potential of carboxylated CdSe/ZnS QDs and Cd single was comprehensively evaluated during short- and long-term experiments using rainbow trout at early developmental stages.
2. The tested CdSe/ZnS QDs in incubation water were found to be chemically stable, because metals were not released from QDs structure and did not cause MT induction.
3. It was first established that the tested QDs formed aggregates in incubation water and agglomerates on the surface of embryos (chorion) and larvae (gills region).
4. It was first shown that QDs do not penetrate into embryos because they get stuck in the chorion, and QDs clog chorion pores thereby damaging its integrity.
5. Application possibilities of confocal fluorescence microscopy, spectroscopy and histology methods were extended to the study of QDs accumulation, penetration and distribution in living and non-living fish embryos and larvae.
6. Results of experiments with Cd and environmental organic and inorganic nano- and micro-scale materials showed that the impact of chemically stable QDs on fish at their early development stages was of mechanical nature.

7. Theoretical and practical significance

Theoretical:

1. The study of prolonged toxicity of CdSe/ZnS QDs and Cd to fish at early stages of development was evaluated using complex methods, and it is an important step in deepening the understanding of metal-based NPs impact mechanisms.
2. The results obtained provide new knowledge on the embryotoxicity and nanotoxicity of QDs to aquatic organisms.
3. The study of QDs accumulation and distribution in fish at early development stages provided new data on the structure and function of the chorion of fish embryos, and helps to understand the mechanisms of

NPs' penetration through biological barriers into other organisms and humans.

4. The visualization of QDs and live embryos and larvae using confocal fluorescence microscopy has pushed back the frontiers of knowledge not only about QDs detection, but also about the accumulation and penetration of other NPs into organisms.
5. The results obtained are useful for a better understanding of the association between the physico-chemical properties of NPs and their impact on organisms.
6. Results of the experiments on fish and environmental nano- and micro-scale materials allow presuming that QDs act in a mechanical way and these findings can be used for explaining the possible impact mechanisms of QDs and other NP aggregates on aquatic organisms.
7. The data obtained can be used for the development of new fields in nanotechnology and nanotoxicology.

Practical:

1. Results of this study will prove useful in solving ecotoxicity problems of QDs and metals.
2. The data derived from the current experimental study will serve as a prerequisite for testing natural water bodies and predicting the possible impacts of QDs and other NPs on aquatic organisms.
3. The data derived from this study are valuable for assessing the ecotoxicological status of the aquatic ecosystem and improving the integrated assessment system of wastewater in Lithuania.
4. The results obtained can be used for the development of environmentally safe NPs.
5. The data obtained from this study will prove useful in regulating and standartizing NPs.

8. The aim and objectives of the thesis

The aim of the study was to investigate the toxicological potential of carboxylated CdSe/ZnS QDs and Cd single, to determine QDs stability, accumulation, penetration, distribution and to explain mechanisms of QDs impact on fish at early development stages.

Study objectives:

1. To investigate and to compare the impact of QDs and Cd single on rainbow trout embryos and larvae during short- and long-term experiments;
2. To examine the chemical stability and behaviour of QDs in incubation water;
3. To determine interactions between QDs and fish embryo surface, and QDs abilities to penetrate through the chorion of embryos;
4. To detect QDs accumulation and distribution in rainbow trout, zebrafish and pearl gourami embryos and larvae;
5. To evaluate the data obtained and to explain mechanisms of QDs impact on fish at early development stages.

9. Statements to defend

1. Carboxylated CdSe/ZnS QDs and Cd single impact on embryos and larvae of rainbow trout during short- and long-term experiments.
2. QDs are chemically stable (there is no metal leakage from QDs).
3. QDs are prone to aggregate in incubation water and to agglomerate on the surface of test-organisms.
4. QDs do not penetrate through the chorion of embryos.
5. QDs accumulate and distribute on the chorion of fish embryos and external tissues of larval bodies.
6. QDs clog pores and damage the chorion integrity of fish embryos.
7. Effects of QDs on test-organisms are attributable to their mechanical impact.

MATERIALS AND METHODS

The simplified scheme of the experimental design and the most important methods used within the framework of this thesis are presented in Figure 1. More details are given in each publication (Table 1; Paper I–XI).

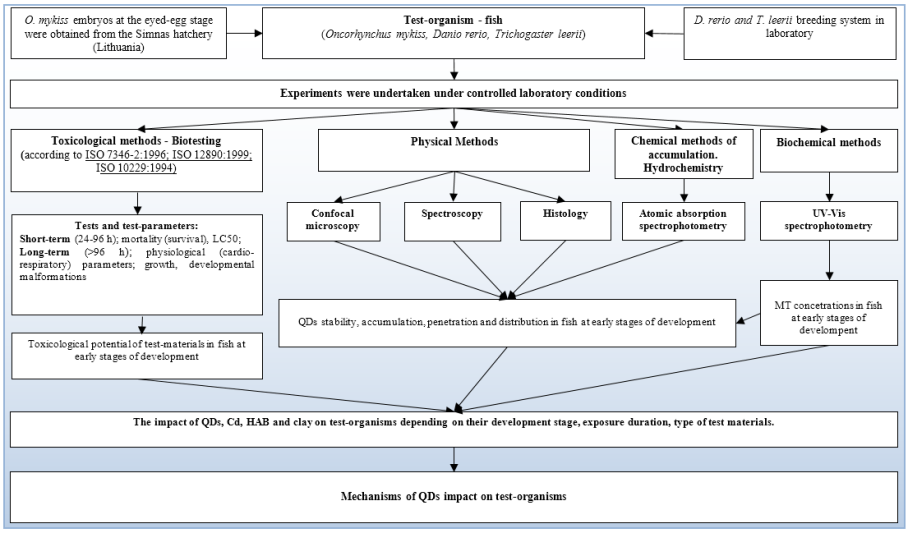

Fig. 1 Scheme of the thesis experimental design.
1 pav. *Disertacijos tyrimų schema.*

For investigations of NPs toxicity, penetration and translocation abilities in fish embryos and larvae, commercially available semiconductor QDs (CdSe/ZnS-COOH cat. No. A10200) were purchased from Life Technologies (USA) (Fig. 2). These QDs have a strong photoluminescence (PL) in red spectral region, with a PL peak of 625 nm. The volume of 100 µL of 8 µM of QDs (stock solution) was dissolved in deep-well water to achieve the final QDs concentration of 4 nM in incubation water (water in which fish embryos and larvae were incubated) (Table 1).

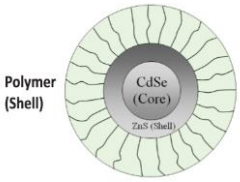

Fig. 2 Structure of CdSe-ZnS quantum dots with a polymer shell structure (Long et al., 2014).
2 pav*. CdSe-ZnS kvantinių taškų su polimeriniu dangalu struktūra (Long et al., 2014).*

Analytical grade cadmium chloride ($CdCl_2 \cdot H_2O$) («REACHIM», Russia) (0.5; 1.0; 2.0; 4.0 and 8.0 μg/L) was used as a toxicant, and stock solutions were prepared by dissolving the necessary amount of salts in distilled water (Table 1).

Experiments with such environmental nano- and micro-scale materials as homogenized cyanobacterial bloom biomass (HAB) (as organic material) (0, 12.5, 25, 50, 100 and 200 mg dw/L) and clay (as an inorganic material) (0, 0.375, 0.750, 1.5, 3.0, 6.0 and 12.0 g/L) were done to explain the possible mechanisms of QDs impact on test-organisms (Table 1; Fig. 3). It was determined that at the beginning of the experiment, the size of QDs was 50–100 nm, that of HAB particles – 60–700 nm, and that of clay particles – 90–1920 nm.

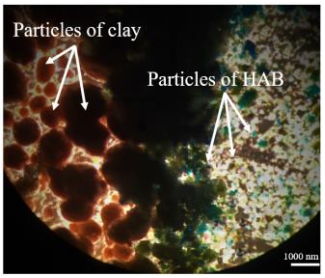

Fig. 3 The comparison of nano- and micro-size particles of cyanobacterial biomass (HAB) and those of clay.
3 pav. *Melsvabakterių biomasės (HAB) ir molio dalelių dydžio palyginimas.*

The toxicological methods employed for studying fish at early stages of development are described in detail in Paper I–XI. The physical methods used to investigate QDs penetration, distribution and accumulation in fish embryos and larvae are described in Papers I, III and VI. The chemical methods employed to investigate Cd accumulation using atomic absorption spectrophotometry are described in Papers I, VIII and X. The description of the biochemical method employed to measure MT content is presented in Paper X.

The methods for measuring QDs and Cd concentrations in incubation water are described in Papers I–III, VI, VIII and X. The statistical methods used are described in detail in respective papers (I–XI).

The bioassay was carried out under controlled laboratory conditions. The study design (including the fish species used, exposure duration and test-parameters) is given in Table 1. The experiments were done with fertilized fish eggs (embryos), larvae and juveniles of rainbow trout (*Oncorhynchus mykiss* (Walbaum 1792)). Additionally, the experiments were carried out

with embryos of pearl gourami (*Trichogaster leerii* (Bleeker 1852)) and zebrafish (*Danio rerio* (F. Hamilton 1822)). Experiments with test-organisms were performed in three replications (N=20 or N=30 in each group). The experiments were conducted in the spring of three years. Artificially fertilized rainbow trout embryos at the eyed-egg stage (at stage 20 of embryogenesis (Ballard 1973)) were obtained from the Simnas hatchery (Lithuania). Effects of test-materials on biological parameters of the test-organisms were investigated (Table 1).

Table 1. The information about performed experiments.
1 lentelė. *Informacija apie vykdytus eksperimentus.*

Fish species	Stage of development	Exposure duration, days	Test-parameters	Test-materials	Paper
Oncorhynchus mykiss	Embryos	4	Mortality	Cd	V
				HAB, QDs, HAB+QDs	–
			Accumulation	QDs, Cd	VIII
		8	Mortality, HR	Cd	VII
				Clay	I
		12	Mortality, HR, QDs stability, accumulation, penetration and distribution	QDs	I, VI
	Larvae	4	Mortality, GVF, HR, behavioural response	QDs, Cd	II, V
			Mortality, GVF, HR, behavioural response, hatching rate	Cd	VII
		8	GVF, HR,	Cd	XI
		10	Mortality GVF, HR, Cd accumulation, MT content	QDs	X
			Accumulation	QDs, Cd	X
		14	Mortality, GVF, HR, relative body mass increase, developmental malformations, behavioural response, QDs accumulation	QDs	III
	Juvenile	4	Mortality	HAB	IV, IX
Danio rerio	Embryos	3	QDs accumulation, penetration and distribution	QDs	I
	Larvae	4	Mortality	HAB	–
Trichogaster leerii	Embryos	1	QDs accumulation, penetration and distribution	QDs	I

„–“ unpublished

RESULTS

The main results of the study are presented in this section. The detailed results are given in each publication and in conference abstracts (Papers I–XI, Conference abstracts I–V).

1. Impact of QDs and Cd single on embryos (Papers VI and VII)

Toxicity of CdSe/ZnS-COOH QDs and Cd to rainbow trout at early stages of development was tested. The bioassay testing was conducted with the goal of exploring the interrelations between different chemical substances (QDs and Cd), exposure duration and biological effects. Biological responses to exposure such as embryo mortality (%) and HR (counts/min) were assessed. The results obtained showed that QDs (4 nM) do not induce a significant increase in mortality of embryos compared to the controls (Table 2; Paper VI: Fig. 2A). Meanwhile, the concentration of 2 μg Cd/L was found to induce a significant increase in embryo mortality as compared to controls (Table 2; Paper VII: Table 2). A significant difference between QDs- and Cd-induced mortality rates was established after 8 and 12 days of exposure (Table 2).

Table 2. Effect of QDs (4 nM) and Cd (2 μg Cd/L) on mortality (%) of rainbow trout embryos depending on exposure duration. Data are reported as Mean ± SD, two-way ANOVA test. The effect significantly different from the control: a, $p < 0.05$. Significant difference between the effects induced by different exposure duration ($p < 0.05$): b – significantly different from the 8 day-exposure effect; c – significantly different from the 12 day- exposure effect. Significant difference between effects of QDs and Cd: d.

2 lentelė. *KT (4 nM) ir Cd (2 μg Cd/L) poveikis vaivorykštinio upėtakio embrionų mirtingumui (%), priklausomai nuo poveikio trukmės (vidurkis ± SD, dvifaktorė ANOVA). Reikšmingai skiriasi nuo kontrolės: a, p < 0,05. Reikšmingai skiriasi tarp poveikio trukmių (p < 0,05): b – reikšmingai skiriasi nuo 8 parų poveikio; c – reikšmingai skiriasi nuo 12 parų poveikio. KT poveikis reikšmingai skiriasi nuo Cd poveikio: d.*

	Exposure, day			
	1	**4**	**8**	**12**
Control	0.0±0.0	0.0±0.0	5.2±0.6	5.2±0.6
QDs	3.5±3.1	3.5±3.1	6.9 ± 2.7^{d}	6.9 ± 2.7^{d}
Cd	6.4 ± 1.1^{abc}	6.4 ± 1.1^{abc}	9.1 ± 2.8^{a}	15.5 ± 1.7^{a}

The test showed that QDs induced HR changes in rainbow trout embryos. The HR of QD-exposed embryos for 1 and 4 days was significantly lower than that of the control group (Fig. 4; Paper VI: Fig. 2B). However, after 8 and 12 days of exposure, the HR of exposed embryos was not found to differ significantly from the control group. Exposure to Cd/L at the concentration of 2 µg significantly decreased the HR of embryos (Fig. 4; Paper VII: Table 2). It was determined that the HR of embryos after 1 and 4 days of exposure to QDs and Cd significantly differed from the HR after 12 days of exposure (Fig. 4).

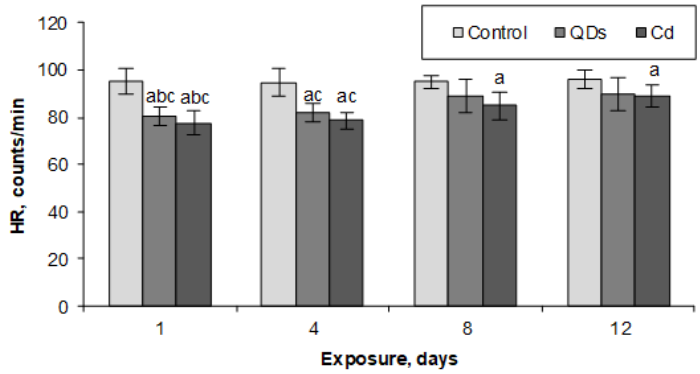

Fig. 4 The effect of QDs (4 nM) and Cd (2 µg Cd/L) on the heart rate (HR, counts/min) of embryos depending on exposure duration. Data are reported as Mean ± SD, two-way ANOVA test. Significant difference from the control: a, $p < 0.05$. Significant difference between the effects induced by different exposure duration ($p < 0.05$): b – significantly different from the 8-day exposure; c – significantly different from the 12 day-exposure.

4 pav. *KT (4 nM) ir Cd (2 µg Cd/L) poveikis vaivorykštinio upėtakio embrionų širdies darbui (ŠD, krt./min), priklausomai nuo poveikio trukmės (vidurkis ± SD, dvifaktorinė ANOVA). Reikšmingai skiriasi nuo kontrolės: a, $p < 0,05$. Reikšmingai skiriasi tarp poveikio trukmių ($p < 0,05$): b – reikšmingai skiriasi nuo 8 parų poveikio; c – reikšmingai skiriasi nuo 12 parų poveikio.*

2. Impact of QDs and Cd single on larvae (Papers II and III)

Significant effects of QDs and Cd after 24 and 96 hours of exposure on mortality, GVF, HR and behavioural response of rainbow trout larvae depending on the type of chemical substances, duration of exposure and

development stage of the affected organism (embryos or larvae) are shown in Paper II: Fig. 1A – D.

QDs were not found to induce significant changes (compared to the control) in mortality and GVF of larvae in the first test, wherein embryos were affected 24 hours before hatching (Paper II: Fig. 1A and B). However, QDs were observed to induce significant increases (compared to the control) in mortality and GVF of larvae at the end of the second test, wherein 1-day-old larvae were exposed to QDs. Meanwhile, the 96 hour- exposure of rainbow trout larvae to Cd induced a significant increase in mortality and a significant decrease in GVF at the end of both tests compared to the control (Paper II: Fig. 1A and B).

QDs were not found to induce a significant decrease in HR of larvae in the first test, but a significant decrease in HR (compared with the control) was recorded in larvae of the rainbow trout exposed to QDs (the second test) and Cd (both tests) (Paper II: Fig. 1C). The 24- and 96-hour exposure to QDs and Cd induced significant changes (compared to the control) in behavioural responses (individuals stopped making nests) in both tests (Paper II: Fig. 1D).

It was found that changes in GVF of the exposed rainbow trout larvae were dependent on the type of the chemical substance they were exposed to and the developmental stage of the affected organism (Paper II: Fig. 1B). Significant differences were found in mortality rates of the larvae exposed to different chemical substances (QDs and Cd) at the end of the both tests, in GVF at the end of the first test and at the beginning of the second test, in HR at the end of both tests, and in behavioural responses only at the end of the second test (Paper II: Fig. 1). In addition, it was established that GVF and HR of the larvae exposed to QDs for 96 hours in the first test depended on the affected organism's developmental stage, while GVF and HR of the larvae exposed to QDs in the second test depended on exposure duration. Furthermore, mortality and GVF of the larvae exposed to Cd were found to significantly vary during all the tests and to depend on exposure duration and the affected organism's developmental stage. These data showed that the impact of Cd on rainbow trout larvae was stronger than on embryos (Paper II: Fig. 1).

The analysis of QDs toxicity results obtained after 14 days of exposure showed a significantly increased mortality, GVF and behavioural responses of larvae as compared to the control (Paper III: Table 2). Meanwhile, after 1 and 4 days of exposure, mortality of larvae did not differ significantly from the control, which was also the case with GVF of larvae after 4 days of

exposure. Additionally, mortality and developmental malformations of larvae after 1 day of exposure, and GVF after 1 and 4 days of exposure were significantly different from those after 14 days of exposure. The 14-day exposure of larvae to QDs induced a significant decrease in RBMI but did not cause a significant increase in HR (Paper III: Table 2).

3. QDs stability (Paper I)

Images of rainbow trout embryos in incubation water (QDs concentration was 4 nM) at the beginning of incubation, and at the end of the experiment (after 10 days of incubation) are presented in Paper I: Fig. 1. At the beginning of the experiment, the incubation water was transparent and homogeneous (Paper I: Fig. 1A), and the homogeneous red PL of QDs detected under blue light illumination (Paper I: Fig. 1C) indicated that suspended QDs in the incubation water were distributed homogeneously.

After 10-day exposure to QDs, huge orange/brown-coloured nonhomogeneous sediments were observed to cover the bottom of the incubation tank and embryos (Paper I: Fig. 1B). On the 10^{th} day of incubation, the different grain size sediments floating in the incubation water and covering embryos were observed to exhibit the red/pink-coloured PL of QDs under UV irradiation (Paper I: Fig. 1D), which indicated that QDs were forming big structures with slightly different optical properties. After prolonged incubation, the widening of the PL band of QDs (from 25 nm at the beginning of the incubation to 30 nm at the end of it) and the negligible shift of the QDs PL band maximum (from 625 nm to 628 nm) were detected (Paper I: Fig. 1E).

4. QDs accumulation and distribution in fish embryos (Paper I)

Additional information about aggregation and/or agglomeration of QDs was obtained from Cd concentration measurements which were performed in the incubation water at the top and bottom of incubation tanks at the end of the experiment. The content of Cd measured in the incubation water as well as in the embryos reflects changes in QDs concentration and distribution over time in volume. Almost all amount of Cd was found in the sediment with only less than one percent of Cd detected in the incubation water collected from the top of the tank (Paper I: Fig. 1G). This fact indicates that all QDs formed aggregates/agglomerates and were distributed in the bottom layer incubation water.

The estimations of Cd concentration in embryos (10 individuals per replicate) made during experiments were analysed in three replications. The values of Cd concentrations measured in rainbow trout embryos after 1- and 4-day exposure to 4 nM of QDs are presented in Paper I: Fig. 1F. The extremely high concentrations of Cd detected in embryos after 1 day-incubation suggest that many QDs were in contact with the biological material. However, the concentration of Cd in contact with embryos after 4-day incubation decreased more than 100-fold, which indicates that after this period of incubation, QDs start separating from the surface of embryos (Paper I: Fig. 1C). Dark embryos and red fluorescing pellets floating around them were lying at the bottom of the incubation tank.

The confocal fluorescence imaging indicated that QDs (red colour representing the PL of the QDs) were located on the surface of zebrafish and pearl gourami embryos (bright field image of embryos after exposure to QDs) (Paper I: Fig. 2A and C). A stack of 2D images taken at different focal planes along the z-axis (Paper I: Fig. 2A) clearly showed that the red photoluminescence of QDs was distributed around zebrafish embryos and could be inserted into the chorion. The pearl gourami embryos after 1-day exposure to QDs and the chorion of embryos after hatching were visualized (Paper I: Fig. 2C (images 3–6)). The red photoluminescence of QDs attributable to the chorion was detected, however, no red photoluminescence of QDs was noted inside the hatched embryos.

Only a small part of a rainbow trout embryo was imaged because it is relatively large in size for confocal imaging (Paper I: Fig. 2B). Nevertheless, the stack of 2D images taken at different focal planes along the z axis clearly showed that the red photoluminescence of QDs was distributed in external embryo tissues (white dashed circles) (Paper I: Fig. 2B), no red photoluminescence of QDs being detected inside the embryo body.

To deepen the understanding of the distribution of QDs in the chorion of embryos, 3D reconstructions were made from the stack of 2D images, which were obtained from different optical sections of the specimen. The 3D autofluorescence of the surface tissues of zebrafish (Paper I: Fig. 3A) and rainbow trout (Paper I: Fig. 3B) embryos formed a pan-like structure because of the spherical shape of the embryo with the bottom flattened by its weight. The vertical cut-off of the iso-surface clearly showed QDs photoluminescence inside the autofluorescence of the 3D iso-surface (Paper I: Fig. 3). Furthermore, due to the relatively small embryo size, a significant PL signal coming from the internal region of the embryo was observed in the inner side of the whole pan-like iso-surface structure. As a result, some of

the embryo's internal structures are presented as a set of irregularly distributed green structures. However, there was no QDs photoluminescence detected in this region (Paper I: Fig. 3). Our data show that QDs photoluminescence (red colour) was detectable only in the autofluorescence region of external embryo tissues (green colour) (Paper I: Fig. 3).

In addition, to examine the incorporation of QDs into the chorion, embryo slices were investigated histologically (Paper I: Fig. 6) and compared with the 3D reconstruction view of live embryos (Paper I: Fig. 2 and 3). The histological view of the rainbow trout embryos exposed to QDs for 8 days showed the presence of QDs only on the surface of the embryo chorion (Paper I: Fig. 6). The histological analysis of the embryo showed the absence of QDs photoluminescence inside the rainbow trout embryo (perivitelline space, yolk sac, etc.) (Paper I: Fig. 6A). The photoluminescence of QDs was detected on the chorion outer layer (Paper I: Fig. 6C, red colour).

The measurements of PL spectrum on histological slices of the fish embryos incubated with QDs (Paper I: Fig. 6D and E) confirmed that the red spots detected in microscopic images were the PL of QDs. The PL spectra (Paper I: Fig. 6D) measured on the outer layer of chorion histological slices (marked as red squares on the microscopic image (Paper I: Fig. 6C) coincided with the QDs photoluminescence spectra measured in the incubation water, proving that the red colour on the chorion surface was PL of QDs accumulated in the chorion outer layer.

5. QDs accumulation and distribution in fish larvae (Papers III and X)

Changes in Cd concentrations recorded in larvae during the experiment are presented in Paper X: Fig. 3. To determine the dependence of QDs accumulation in rainbow trout larvae on exposure duration, samples of larvae were collected after 4, 7 and 10 days of exposure. Cd concentrations in larvae after 4, 7 and 10 days of exposure to QDs significantly differed from those in controls (Paper X: Fig. 3). The maximum value of accumulated Cd was found in the larvae exposed to QDs for 10 days (1.302±0.272 μg/g). However, the concentration of Cd in larvae was not found to depend on exposure duration (Paper X: Fig. 3).

The fluorescence spectroscopy analysis showed that QDs solution was relatively stable in experimental water for 7 days (Paper III: Fig. 1A). Figure 1C (Paper III) shows data on QDs accumulation in the larval structures incubated with QDs at sublethal concentrations. However, after 24 hours of

exposure, QDs photoluminescence in larvae was observed in the head (gills) region with no distinguishable fluorescence in other regions (Paper III: Fig. 1C).

6. Mechanisms of QDs impact on fish at early stages of development (Papers I and III, Conference abstracts IV and V)

Our study revealed that after 1–2 day exposure, there was observed formation of QDs aggregates of different size on the surface of the exposed rainbow trout embryos (chorion) (Paper I: Fig. 4B). QDs or small QDs aggregates formed in the incubation water were observed to attach to the surface of embryos forming aggregation centers (Paper I: Fig. 4B). After 3 and 4 days of incubation with QDs, the number of the aggregates attached to the surface of the chorion increased (Paper I: Fig. 4C). Furthermore, with the increase of incubation time, drastic changes in the outer layer of the chorion were detected. QDs intervened the embryo chorion causing chorion surface disruption (Paper I: Fig. 4D). The disintegration of the outer part of the chorion membrane and separation of QDs agglomerates with the mucus from the embryo were detected after 4–6 days of incubation (Paper I: Fig. 4D). The opening of the chorion (where many QDs aggregates are concentrated) is clearly visible in the 3D reconstructed image of the rainbow trout embryo chorion (Paper I: Fig. 5).

Additionally, the reconstructed 3D view of the chorion revealed its nonhomogeneous autofluorescence (Conference abstract V: Fig. 1C). The high magnification (60-fold) views of these chorion sections supported the assumption that the brighter spots represent pores of the rainbow trout chorion (Conference abstract V: Fig. 1C and D).

The brighter green spots were visible as elongated, irregular cylindrical structures embedded in the chorion up to 3–5 μm deep (Conference abstract V: Fig. 1D). QDs photoluminescence was concentrated within the outer layer composed of cylindrical structures (Conference abstract V: Fig. 1D (images 1–4)). Aggregates of QDs could clog pores of the chorion. The distribution of QDs photoluminescence in the chorion depended on the size of QDs aggregates (Conference abstract V: Fig. 1D (images 1–4)). The photoluminescence of small QDs aggregates was observed in the spatial area of cylindrical elements in the outer layer, i.e. at a depth of 3–5 μm (Conference abstract V: Fig. 1D (images 1–2)). With the increase in the size of QDs aggregates, the photoluminescence of QDs was observed to cover

larger spatial areas and greater depths of the chorion (Conference abstract V: Fig. 1D (images 3–4)).

Although penetration and accumulation of QDs inside embryos were not observed, there were several damaged embryos detected. Figure 7 (Paper I) shows abnormally developing embryos in QDs incubation water. The present study demonstrated that QDs induce spinal curvature, blood clots and head hatching (Paper I: Fig. 7). Also, this study showed that QDs exposure causes blood clots in the heart area of embryos (Paper I: Fig. 7).

Additionally, this study revealed such QDs exposure-induced malformations in fish larvae as blood clots in the head area and yolk sac (Paper III: Fig. 2A and B).

It was noticed that QDs cover the chorion of embryos and the gills of larvae like natural nanoscale particles (HAB or clay). Due to their fine-size fractions, HAB and clay produce a negative effect on fish at early development stages and, thus, are examples of negative impact factors on fish. Experiments with HAB and clay (Paper IV: Fig. 4B and Paper I: Fig. 9) were done to make sure that the effects of QDs aggregates on test-organisms are attributable to their mechanical impact (Paper I: Fig. 4 and 5).

The highest concentration of HABs (used in the experiments as examples of organic nano- and micro-scale materials) causing 100% mortality was 200 mg dw/L and the concentrations causing no mortality were 12.5 and 50 mg dw/L (Paper IV: Fig. 4B). The effect of HABs on mortality of test-organisms was rather ambiguous, with no clear pattern or consistency (Paper IV: Fig. 4B).

The results obtained from the experiments involving clay treatment indicated that with the increase of the dosage and exposure, rainbow trout survival did not decrease significantly compared to that of the control group (Paper I). However, a slight decrease in survival was observed after 4 and 8 days of exposure. After 4-day exposure to clay at 6.0 g/L concentration, 9 embryos from three replicates (N=90) died, and after 8-day exposure to clay at 12.0 g/L concentration, 10 embryos from three replicates (N=90) were found dead (Paper I). In addition, the experiments revealed that 1- and 4- day exposure to clay induced bradycardia in fish embryos, but later (after 8 days of exposure) HR recovered and reached that of the control group (Paper I: Fig. 9).

The content of MT was used as a marker of internal QDs exposure (Paper X). However, MT contents in larvae showed no significant changes after 7- and 10-day treatment with QDs compared to the control (Paper X: Fig. 4).

DISCUSSION

The rapid increase in nanomaterial production heightens the need to understand toxicological potentials of NPs and their impact on health, safety and the environment. The data obtained from this study showed that mortality of the rainbow trout embryos exposed to QDs (4 nM) in the prolonged experiment remained statistically unchanged, i.e. the number of dead embryos was not significant (Table 2). However, exposure of rainbow trout embryos to 2 µg/L concentration of Cd induced a relevant ($p < 0.05$) increase in their mortality (Table 2). The concentration of 2 µg Cd/L was chosen according to the 96 hours LC50 value for rainbow trout larvae (Paper V: Table 1). Zhang et al. (2012a; 2013b) indicated that the value of 120 hours LC50 for zebrafish larvae is 1.98 mg/L of CdSe-MPA QDs, 185.9 nM of CdTe-TGA QDs and 22.31 mg/L of CdTe-TGA QDs. The findings of our study support previous research (Eaton et al., 1978; Levit 2010; Zhang et al., 2012a; Calfee et al., 2014) regarding an increase in mortality of fish at early development stages after exposure to low Cd concentrations (ranging from 2 µg/L to 12 µg/L). Earlier research also showed that exposure to the concentration of 100 µg Cd/L completely inhibited development of *Oryzias javanicus* embryos and caused 100 % mortality (Ismail and Yusof 2011). Mortality of fish embryos and larvae is additionally affected by metals leaking from QDs structures (Hardman 2006; Rocha et al., 2017). A large amount of water-soluble Cd, especially Cd at high concentrations, is likely to penetrate into embryos and accumulate around eggs, and finally lead to their death (Annabi et al., 2013).

Our results showed that HR of the embryos exposed to QDs for 1 and 4 days was significantly lower compared to that of controls (Fig. 4). In this study, a significantly decreased HR of embryos compared to that of the control group was also observed after 1–12 days of exposure to Cd (Fig. 4). It was found that a significant decrease in HR of the embryos exposed to QDs and Cd was exposure duration dependent. However, with a further increase of exposure time, the HR of QDs-exposed embryos recovered to the HR of the control group. This fact indicates that the HR of QDs-exposed fish embryos can recover. Witeska et al. (2014) described the recovery of the Cd-exposed fish at early developmental stages.

Results of this thesis demonstrate that short-term (24 and 96 hours) exposure to sublethal concentrations of QDs and Cd increases mortality, affects functions of the cardiorespiratory system and elicits behavioural response in rainbow trout larvae (Paper II: Fig. 1). The current study

revealed that Cd was more toxic to larvae than QDs, that exposed larvae were more sensitive to Cd and QDs than exposed embryos, and that longer duration of (96 hours) QDs and Cd exposure induced marked changes in test-parameters (Paper II: Fig. 1).

During the long-term (14 days) toxicity test, QDs at sublethal concentrations were found to produce the following negative effects on rainbow trout larvae: increased mortality and GVF, disrupted larval growth, malformations, and changes in behaviour (Paper III: Table 2). The tests revealed that QDs toxicity effects intensified with an increase of exposure duration. Similar results were obtained from some studies examining exposure of fish embryos and larvae to QDs (King-Heiden et al., 2009; Duan et al., 2013). Cd and CdSe/ZnS QDs were also observed to produce similar effects on larval mortality, respiratory changes and behavioural responses during the short-term test (King-Heiden et al., 2009; Duan et al., 2013; Zolotarev et al., 2013). Duan et al. (2013) showed that exposure to CdTe QDs caused an increase in mortality of zebrafish embryos and larvae in a dose- and time-dependent manner. Furthermore, QDs toxicity was found to be influenced by the QDs coating and to be more potent in causing mortality than an equivalent amount of Cd^{2+} (King-Heiden et al., 2009).

Our data showed that exposure to QDs elicited significant behavioural responses in fish (exposed individuals stopped making nests) (Paper III Table 2). Some authors (Zolotarev et al., 2012; Duan et al., 2013) also noted changes in behaviour of the QDs-exposed fish larvae. Duan et al. (2013) performed two toxicological tests to determine the prolonged effects of CdTe-thioglycolic acid QDs on the locomotor behaviour of zebrafish larvae and the effects of light-dark cycles on zebrafish larvae behaviour. Zolotarev et al. (2012) noticed that after 6 and 7 days of incubation with CdSe/CdS/ZnS/S,S-dihydrolipoic acid/polyacrylic acid QDs, zebrafish larvae exhibited poor swimming coordination and low locomotor activity. Moreover, the authors detected the malformation of a swimming bladder, which could have altered activity of larvae.

In this study, Cd was selected as an element capable of inducing the negative effects that are comparable with those of Cd-based QDs. King-Heiden et al. (2009) found that 2-28 μM Cd could be released from CdSe/ZnS QDs coated with PEG after 120 hours. In our study, the concentration of Cd within the QD structure was 10^{-6} M and in $CdCl_2$ solution it was 1.8×10^{-8} M, i.e. the Cd concentration in QDs suspension was 56 times higher than in $CdCl_2$ solution. Therefore, in the event of QDs

degradation and metal leakage from the QDs structure, the impact of Cd on test-organisms can be lethal.

Our study demonstrated that tested CdSe/ZnS QDs (4 nM) were aggregating in incubation water and were forming agglomerates on the surface of fish embryos throughout the period of 1–12 days. The recorded widening and negligible shift of QDs PL band maximum during the incubation period (Paper I: Fig. 1E) could be explained by the interaction and aggregation of QDs (Kulvietis et al., 2011). Changes in hydrodynamic radius, aggregation and/or agglomeration of QDs in aqueous media were mentioned in some papers investigating QDs interaction with aquatic organisms (Gagné et al., 2008; King-Heiden et al., 2009; Zolotarev et al., 2012). King-Heiden et al. (2009) studied the toxicity of CdSe/ZnS QDs functionalized with a different coating to zebrafish embryos and larvae. The authors noted changes in QDs hydrodynamic diameter indicating their possible aggregation (King-Heiden et al., 2009). Zolotarev et al. (2012) also noticed QDs coagulation in solution (got stuck to each other), and deposition of QDs on the surface of zebrafish embryos after 1 or 2 days of incubation. These findings are in accordance with the results of this thesis.

The extremely high concentrations of Cd recorded in embryos after 1-day incubation suggest that many QDs are in contact with the biological material (Paper I: Fig. 1F). However, the concentration of Cd in contact with embryos after 4-day incubation decreased more than 100-fold. This fact indicates that after 4 days of incubation, QDs were separating from the embryo surface (Paper I: Fig. 1C). Dark embryos and red fluorescing pellets floating around them were lying on the bottom of the incubation tank. This fact clearly shows that a decrease in Cd concentration in embryos is attributable to the formation of agglomerates of QDs and mucus on the embryo surface and their separation from embryos into the incubation water as it is seen in Figure 4D (Paper I). It seems that QDs did not penetrate into the embryo.

In addition, the analysis of the total Cd concentration in embryos revealed a decrease after 4-day embryo treatment with QDs (Paper I: Fig. 1F), which implies that the test-organism has some sort of protection mechanisms for removing QDs from its surface. However, the chorion in these surface areas lost solidity and QDs aggregates induced defragmentation and damage to its outer layer (Paper I: Fig. 4E). However, these processes require further studies. Meanwhile, NPs could interact with proteins on and/or in cells, resulting in altered protein conformation, disruption of the plasma membrane integrity, and production of ROS (Tsoi et al., 2013; Strtak et al., 2017).

The determination of chemicals concentrations in embryos and larvae is a challenging procedure because the tiny sample amount (1 larvae ~ 0.1 g) requires highly sensitive analytical techniques. In this study, larvae were not feeding, which implies that the only possible way for QDs to penetrate into larvae was skin-absorption. It is well known that biological barriers play a significant role in determining QDs biodistribution (Chu et al., 2010). The small size of QDs (between 1 and 100 nm) permits them to get into the body through cellular barriers, reach organs and tissues and interact with biological structures, thus impacting on normal functions in different ways (Maldiney et al., 2011). Upon entry into the larval body through skin-absorption, NPs can selectively accumulate in the head, yolk sac or in the tail (Kang et al., 2015). However, QDs can be eliminated in the urine of larvae or can be degraded into particles and removed by lysosome-like vesicles, and then accumulate in the kidney and liver (Lei et al., 2011). In contrast, during normal metabolism, it is the liver and kidneys that are the primary tissues of Cd accumulation (Haouem et al., 2007). Lei et al. (2011) noted that MAA-QDs were unable to diffuse into the yolk of larvae because of the high content of lipids in yolk cells.

However, data on the association of QDs with rainbow trout embryos and larvae are scarce. In recent studies, zebrafish embryo and larvae were visualized mainly by using the electron microscopy (Ag NPs, Asharani et al. (2008), fluorescence microscopy (SiO_2 NPs, Fent et al. (2010); Co_3O_3, CuO, NiO, ZnO NPs, Lin et al. (2011)), inductively coupled plasma mass spectrometry (Al_2O_3, Ag and Au NPs, Böhme et al. (2017); ZnO NPs, Brun et al. (2014); Cu_2O, $CuCl_2$ NPs, Chen et al. (2011)), and intravital multiphoton laser scanning microscope (CdSe/ZnS QDs, Lee and An (2014)). Various visualization techniques were not used for studying damage to fish embryo chorion. However, cell necrosis in the blastoderm and in the yolk syncytial layer of zebrafish at early life stages was revealed in other studies (Osborne et al., 2013).

This study proved that confocal fluorescence microscopy is a valuable tool for detecting and visualizing CdSe/ZnS QDs in test-organisms such as rainbow trout, zebrafish and pearl gourami embryos. Based on 2D images, QDs photoluminescence was detected in external tissues of zebrafish, rainbow trout and pearl gourami embryos, but not in the central part of these embryos (Paper I: Fig. 2). This fact proves that in this study QDs aggregates did not penetrate into the chorion after 10–12 days of incubation. Additionally, the results obtained show that rainbow trout chorion consists of

three different light transmission layers and that QDs accumulate in the first layer of the chorion (data not published).

The internal surface of fish chorion is permeated with many pores (Henn 2011). According to Cheng et al. (2007), some chemicals and small NPs can penetrate through these pores. The diameter of zebrafish pores is 0.5–0.7 μm, the center-to-center distance is 1.5–2.0 μm (Rawson et al., 2000). Our data showed that the diameter of pores on the outer surface of the rainbow trout embryo was approximately 1.1±0.2 μm and the center-to-center distance was around 2.2±0.3 μm (Conference abstract V: Fig. 1C).

The detailed examination of QDs distribution in rainbow trout embryos proved that pores are the predominant site for the uptake and accumulation of QDs aggregates (Conference abstract V: Fig. 1D). The presence of QDs inside the exposed embryos was not detected. However, aggregates of QDs can clog pores of the chorion (Conference abstract V: Fig. 1D), thereby, according to Celá et al. (2014), disrupting the transport of oxygen and carbon dioxide through the chorion.

The mechanism of QDs impact on fish during their sensitive embryonic period is not clear (King-Heiden et al., 2009, Duan et al., 2013, Zolotarev et al., 2013). Mechanisms of organisms in response to prolonged QDs exposure require further research. Most of the studies performed indicate that toxicity is linked to QDs instability and metal leakage. MT contents (an indicator of metal ion exposure) were used to detect Cd^{2+}-induced toxicity. However, MT contents in larvae were not found to significantly increase after 7 and 10 days of exposure (Paper X: Fig. 4). The data obtained from this study are in agreement with those reported by Fischer et al. (2006), indicating that the ZnS shell and surface ligands protect QDs from degradation *in vivo*. Therefore, QDs were stable during 10 days of exposure and QDs absorption in larvae was not recorded. In contrast, King-Heiden et al. (2009) noticed that QDs degraded *in vivo* at least partially, MT expression correlated with $CdCl_2$ and QDs exposure concentrations.

In this study, destruction of embryo chorion was detected before embryo hatching (Paper I: Fig. 4E and 7). This finding showed that the damage caused to the chorion integrity by the increasing size and amount of QDs aggregates/agglomerates was mainly due to their mechanical impact.

According to Federici et al. (2007), Smith et al. (2007), Mansouri and Johari (2016), Ostaszewska et al. (2016) and Songe et al. (2016), various stressors induce increased mucus secretion on the surface of fish. The mucous layer has been reported to be a barrier against the penetration of chemicals and bacteria into the organism (Vatsos et al., 2006; Kumari et al.,

2009, Villarreal et al., 2014). Mucus secretion was observed in our study as well. In our opinion, the mucus of rainbow trout embryos interacts with QDs aggregates/agglomerates. During the prolonged experiment, the complex of QDs and mucus was noted to separate from the chorion surface and damage its integrity (Paper I).

Additionally, aggregates of QDs can clog chorion pores (Paper I), which can affect nutrient transportation and produce negative effects on embryonic development (Ninness et al., 2006; Celá et al., 2014; Jaramillo et al., 2015). Our study showed some QDs exposure-induced malformations in fish at early development stages (Paper I: Fig. 7; Paper III: Fig. 2). QDs induced spinal curvature, head hatching of embryos (Paper I: Fig. 7) and blood clots in the heart area of embryos (Paper I: Fig. 7B). Larvae exposed to QDs were also found to possess developmental malformations (Paper III: Fig. 2A and B). Pericardial edema, which is the main type of QDs-induced malformations, can affect embryo cardiac function (Duan et al., 2013). There are no data about QDs-induced malformations in rainbow trout embryos. In zebrafish embryos, QDs induced such developmental malformations as eyespots, melanin developmental inhibitions (Zhang et al., 2012b), and disintegrated embryos (George et al., 2011; Duan et al., 2013). According to Ługowska and Sarnowski (2011), head hatching is less successful as compared to tail hatching. In addition, head hatching usually causes death and/or body malformations of larvae. A decrease in zebrafish hatching rate after exposure to QDs was reported by King-Heiden et al. (2009), George et al. (2011), Zhang et al. (2012a, 2013b), Zolotarev et al. (2012) and Duan et al. (2013).

QDs can physically clog the pores (Conference abstract V: Fig. 1) of the embryo chorion like such environmental nano- and micro-scale materials as cyanobacterial biomass (HAB) (Wyatt et al., 2010; Wu and Zhou 2012; Gao 2016) and clay (Julien and Bergeron 2006). The negative effects of these materials on aquatic organisms are comparable with those produced by QDs.

Our results showed that QDs aggregates like particles of HAB biomass and clay accumulated on the surface of test-organisms and did not penetrate through the chorion of fish (Papers I and IV). The amount of biologically active compounds (such as cyanotoxins) in HAB biomass was rather low (Paper IV: Table 1). The data obtained showed that exposure to the highest HAB biomass concentration (200 mg dw/L) caused 100 % mortality of rainbow trout juveniles, however, lower concentrations were not found to induce a significant increase in mortality (Paper IV: Fig. 4B). In our opinion, mortality of the fish exposed to the highest concentration of HAB biomass is

attributable to toxicity of biologically active compounds and mechanical impact of biomass particles, whereas physiological changes in the juveniles exposed to lower concentrations of HAB are more likely to be the outcome of the mechanical impact of biomass particles. No mortality was observed in the embryos exposed to the same HAB biomass concentrations and various concentrations of clay, which can be explained by protective properties of the embryo chorion.

However, upon 1- and 4-day exposure to QDs (Paper VI: Fig. 2B), various concentrations of HAB biomass (data not published) and clay (Paper I: Fig. 9), rainbow trout embryos were found to exhibit bradycardia, which was also reported by Zhang et al. (2012b) and Duan et al. (2013). In our opinion, the impact of environmental and engineered nanoscale materials is attributable to the mechanical stress they cause to organisms, e.g. gas exchange (hypoxia), caused by the coverage of the organism surface with particles of materials. This was confirmed by some authors (Lapointe et al., 2004; Grieg et al., 2005; Julien and Bergeron 2006; Louhi et al., 2008; Wyatt et al., 2010; Gao 2016).

Disturbed gas exchange is expected to impact on embryos and larvae (Paper I–III; V–IV; VIII and X). QDs agglomerated on the chorion surface of fish embryos (Paper I: Fig. 1), induced depletion of oxygen exchange and hypoxia (Zhu et al., 2012; Gao 2016). Hypoxia-induced ROS may be a physiological response to oxygen deficiency. However, hypoxia-induced ROS production remains unclear. Several reports have shown that hypoxia is a mutagen, teratogen, because it affects fish embryonic development (Shang and Wu 2004; Shang et al., 2006). Hypoxia is known to induce mortality, malformation, to delay fish (*D. rerio*) embryonic development, hatching, to disrupt the apoptotic pattern and balance of sex hormones (Shang and Wu 2004; Shang et al., 2006; Gao 2016). However, hypoxia inducing development impairments in fish remains unclear (Wu 2009). Nevertheless, such findings are hypothesis-generating and require further detailed studies (Gao 2016).

Summarized and specific results of QDs impact on fish embryogenesis are shown in an empirical model (Fig. 5). These results lead to the conclusion that negative effects of QDs on fish in early development could be related with hypoxia induced by the mechanical impact of QDs.

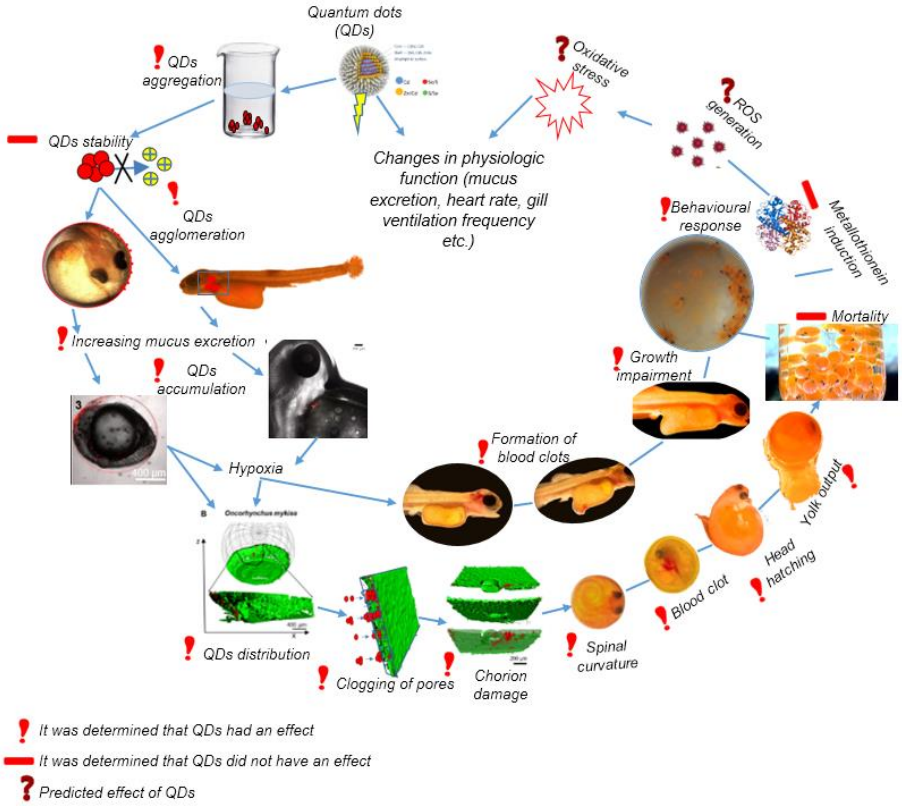

Fig. 5 Empirical model of QDs impact on fish in early development.
5 pav. *KT poveikio žuvims ankstyvajame jų vystymesi empirinis modelis.*

CONCLUSIONS

1. In this study, carboxylated CdSe/ZnS quantum dots (QDs) (4 nM) were not found to affect mortality of rainbow trout embryos, but they were found to cause mortality of larvae during both short- and long-term experiments. In contrast, mortality rates of embryos and larvae increased with increasing duration of exposure to Cd (2 μg/L). In many cases, alterations in biological parameters (respiration, growth, development and behaviour) of test-organisms were related to the duration of exposure to QDs and Cd.
2. It was determined that the impact of QDs and Cd on rainbow trout at early life stages depended on the type of chemical substances and developmental stage of the organism affected. Cd was found to be more toxic to embryos and larvae than QDs. However, affected larvae proved to be more sensitive to Cd and QDs compared to affected embryos.
3. Chemically stable QDs (which do not release Cd) were found to form aggregates in incubation water and agglomerates on the surface of embryos and larvae of the tested fish. QDs accumulated and got stuck in the chorion of fish embryos, while in the case of fish larvae, QDs accumulated and distributed in the region of gills.
4. The performed comprehensive analysis of QDs proved that QDs did not penetrate into the embryos of rainbow trout, zebrafish and pearl gourami. Therefore, it can be stated that the chorion of fish embryos is a protective barrier against QDs penetration. However, during prolonged exposure, damage of the chorion integrity was observed in rainbow trout embryos: ODs penetrated into the chorion, chorion pores clogged and the outer layer of the chorion was disintegrated.
5. The data obtained showed that the effects of QDs on biological parameters of fish at its early development stages were related not to the release of metal from QDs structure, but mainly to other physico-chemical properties of QDs (the ability to form aggregates and agglomerates).
6. Results of fish toxicity experiments with metals and environmental organic and inorganic nano- and micro-scale materials and induction of metallothionein in tissues of fish larvae allow us to presume that the impact of chemically stable QDs on test-organisms is of mechanical nature.

SANTRUMPOS

ŽVD – Žiaunų ventiliacijos dažnis
HAB – Melsvabakterių biomasė
ŠD – Širdies darbas
MT – Metalotioneinai
ND – Nanodalelės
FL – Fotoluminescencija
KT – Kvantiniai taškai
SKMP – Santykinis kūno masės padidėjimas
TGA – Tioglikolinė rūgštis
UV – Ultravioletas

SANTRAUKA

Vis didesnį susirūpinimą kelia aktyvi nanotechnologijų plėtra, kadangi nanodalelės (ND) tapo nauja aplinkos teršalų klase, galinčia pakenkti ekosistemoms ir žmonių sveikatai (Hardman 2006; Blickley *et al*. 2014; Rocha *et al*. 2017; Paper III). Viena iš jų rūšių yra mažos puslaidininkinės ND – kvantiniai taškai (KT), kurie pasižymi išskirtinėmis fizikocheminėmis savybėmis (Alivisatos *et al*. 1996; Gagne *et al*. 2010; Piccinno *et al*. 2012; Paper I).

KT yra koloidinės nanostruktūros medžiagos, sudarytos iš puslaidininkinio metalo šerdies (CdSe, CdTe ir kt.) (Paper I). CdSe šerdis dažnai padengiama ZnS dangalu, kuris veikia kaip šerdies apsauga ir neleidžia Cd atsipalaiduoti bei pagerina KT fotoliuminescenciją (Domingos *et al*. 2011; Zarco-Fernández *et al*. 2016). KT gali turėti organinius apvalkalus, kurie padidina jų tirpumą vandenyje ir padidina jų patekimą į biologinius objektus (Medintz *et al*. 2005; Paper VIII). Dėl išskirtinių savybių ir plataus KT taikymo įvairiose srityse (optoelektronikoje, pramonėje, biomedicinoje) jų pasaulinė produkcija didėja (Piccinno *et al*. 2012; Paper I). Tačiau yra nedaug duomenų apie KT poveikį organizmams ir aplinkai (Rocha *et al*. 2017). Todėl ir kyla nemažai diskusijų dėl jų naudojimo saugumo (Paper I).

KT toksiškumas yra vis didėjanti problema, ypač dėl jų specifinių savybių, fizikinių ir cheminių transformacijų aplinkoje ir toksiškų metalų atsipalaidavimo iš KT šerdies (Ipe *et al*. 2005; Hardman 2006; Ribeiro *et al*. 2012; Katsumiti *et al*. 2014; Devin *et al*. 2016; Rocha *et al*. 2017; Paper I). Pastaraisiais metais žuvų naudojimas nanotoksiškumui vertinti auga eksponentiškai (Chakraborty *et al*. 2016; Rocha *et al*. 2017). Žuvys dėl gana trumpo embrioninio vystymosi yra plačiai naudojamos biomedicininiuose tyrimuose (Powers 1989; Yong *et al*. 2013; Rocha *et al*. 2017). Dryžuotoji danija ir vaivorykštinis upėtakis kaip modeliniai test-organizmai yra plačiai naudojami nanotoksiškumui vertinti (Rocha *et al*. 2017). Taip pat šios žuvų rūšys, ypač dėl jų jautrumo ankstyvosiose vystymosi stadijose, yra plačiai naudojamos standartizuotuose cheminių medžiagų, jų mišinių toksiškumo tyrimuose (ISO 12890: 1999; ISO 7346-1: 1996; ISO 10229: 1994; Hrovat *et al*. 2009; Rocha *et al*. 2017). Kadangi vaivorykštinis upėtakis pasižymi ilgesne embrionų vystymosi trukme nei dryžuotoji danija, todėl galima

vykdyti ilgesnės trukmės toksikologinius eksperimentus su embrionais (Ballard 1973; Vosylienė 2007; Paper I).

Nanotoksiškumui žuvyse vertinti yra naudojami skirtingi rodikliai: ritimosi greitis, organų vystymosi pažaidos, elgsena, imunotoksiškumas, genotoksiškumas, genų raiška, neurotoksiškumas, endokrininės sistemos ir reprodukciniai pokyčiai, mirtingumas (Chakraborty *et al*. 2016; Paper X). Kai kurie autoriai nustatė, kad KT yra toksiški skirtingoms gyvūnų rūšims: *Caenorhabditis elegans* (Qu *et al*. 2011), *Bombyx mori* (Liu *et al*. 2014), *Hydra vulgaris* (Ambrosone *et al*. 2012), *Danio rerio* (Zhang *et al*. 2012a, 2012b), *Onchorchyncus mykiss* (Federici *et al*. 2007; Smith *et al*. 2007; Shahbazzadeh *et al*. 2009; Scown *et al*. 2010; Munari *et al*. 2014) ir pelėms (Chu *et al*. 2010; Scoville *et al*. 2015; Wang *et al*. 2016).

Trumpalaikiuose tyrimuose nustatyta, kad KT sukelia įvairius fiziologinius, biocheminius pokyčius dryžuotosios danijos embrionuose ir lervose (King-Heiden *et al*. 2009; Leigh *et al*. 2012; Zhang *et al*. 2012b; Zolotarev *et al*. 2012; Duan *et al*. 2013; Rocha *et al*. 2017; Paper VI), o jų poveikis vaivorykštiniams upėtakiams ankstyvajame jų vystymesi nebuvo tirtas. Reikalingi išsamesni tyrimai, siekiant išsiaiškinti KT toksikologinį potencialą ir jų poveikio mechanizmus, ypač ankstyvaisiais žuvų vystymosi etapais.

Toksikologiniai tyrimai parodė, kad KT toksinis poveikis žuvims pasireiškia, kai KT skyla ir metalo jonai atsipalaiduoja iš jų struktūros (Gagné *et al*. 2010; Paper II). Remiantis King-Heiden *et al*. (2009) eksperimentais, CdSe/ZnS KT komponentai, tokie kaip Zn, Se ir S, išskyrus Cd, nesukelia reikšmingo toksinio poveikio dryžuotajai danijai ankstyvajame vystymosi etape. Nanotechnologijos kelia taršos Cd padidėjimo riziką vandens ekosistemoms (Rzigalinski, Strobl 2009). Atlikti lauko tyrimai parodė, kad vandens ekosistemos užterštumas Cd ir jo akumuliacija dugno nuosėdose gali išlikti daugelį metų (Baudrimont *et al*. 2005; Coynel *et al*. 2007; Annabi *et al*. 2013; Pereira *et al*. 2015; Paper VIII). Cd toksiškumas išsamiai ištirtas skirtingoms gėlavandenėms žuvų rūšims (Al-Asgah *et al*. 2015). Nustatyti morfologiniai, fiziologiniai, hematologiniai, biocheminiai ir imunologiniai pokyčiai žuvyse ankstyvajame jų vystymesi, paveikus jas Cd druskomis (Brinkman *et al*. 2007; Ismail, Yusof 2011; Heydarnejad *et al*. 2013; Paper VIII). Metalotioneinų kiekis (MT) yra naudojamas kaip biožymuo, leidžiantis įvertinti Cd jonų, atsipalaidavusių iš KT, kiekį *in vivo* (King-Heiden *et al*. 2009). Eksperimento su dryžuotosios danijos embrionais ir lervomis rezultatai parodė, kad KT tik iš dalies skyla, o stebimas KT

toksiškumas nėra būdingas Cd jonų sukeltam toksiškumui (King-Heiden *et al*. 2009; Paper X).

Daugelis mokslininkų analizavo KT toksiškumą, o ne jų kaupimasi, patekimą ir pasiskirstymą žuvų embrionuose ir lervose (Domingos *et al*. 2011; Zhang *et al*. 2012b; Zolotarev *et al*. 2012; Duan *et al*. 2013; Rocha *et al*. 2017; Paper I). Zarco-Fernández *et al*. (2016) tyrimas parodė, kad Cd, esantis druskų pavidalu ir esantis CdSe/ZnS KT, kaupiasi skirtingose dryžuotosios danijos lervos kūno srityse (Paper VIII). Nustatyta, kad Cd kaupimasis vandens organizmuose priklauso nuo jo koncentracijos, patekimo būdo, aplinkos sąlygų ir kitų veiksnių (Karakoç, Dinçer 2003; Bowen *et al*. 2006; Jezierska; Guinot *et al*. 2012). Tuo tarpu KT kaupimasis žuvyse ankstyvosiose jų vystymosi stadijose priklauso nuo KT sudėties, dydžio ir paviršiaus padengimo parametrų (Zarco-Fernández *et al*. 2016; Paper VIII).

Murugan *et al*. (2015) nuomone, ND dėl mažo dydžio gali patekti pro biologines membranas. Nustatyta, kad žuvų embrionų chorionas geba apsaugoti besivystantį organizmą nuo ND poveikio (Kashiwada 2006; Lee *et al*. 2007; Asharani *et al*. 2008; Browning *et al*. 2009; Fent *et al*. 2010; Osborne *et al*. 2013; Kang *et al*. 2015; Rocha *et al*. 2017). Palyginti su kitomis ND, informacijos apie KT patekimą ir pasiskirstymą žuvyse, ypač ankstyvosiose jų vystymosi stadijose, yra nedaug (Zolotarev *et al*. 2012; Zarco-Fernández *et al*. 2016). Daugiausia darbų buvo skirta KT elgsenai dryžuotojų danijų embrionuose ir lervose tirti (Lee *et al*. 2007; Asharani *et al*. 2008; Browning *et al*. 2009; Fent *et al*. 2010; Zolotarev *et al*. 2012; Böhme *et al*. 2015). Tačiau literatūroje neaptikta duomenų apie KT kaupimąsi, jų patekimą ir pasiskirstymą vaivorykštinio upėtakio embrionuose ir lervose. Zolotarev *et al*. (2012) ir Petushkova *et al*. (2015) yra pastebėję dideles KT struktūras dryžuotosios danijos embrionų choriono paviršiuje. King-Heiden *et al*. (2009) nustatė, kad vykstant agregavimui, bandymo vandenyje KT hidrodinaminis skersmuo padidėja. Tačiau iki šiol dar nebuvo išsamiai ištirtas KT agregatų susidarymas, jų kaupimasis ir patekimas į žuvų embrionų vidų pro chorioną.

Pažymėtina, kad nėra duomenų apie kelių fizikinių metodų (spektroskopijos ir mikroskopijos) naudojimą, siekiant vizualiai nustatyti KT kaupimąsi ir pasiskirstymą žuvų embrionuose ir lervose (Paper I). Nors konfokalinė mikroskopija plačiai naudojama biomedicininiuose tyrimuose (Bijeesh *et al*. 2017), tačiau jos taikymas, vertinant KT poveikį ir jų kaupimąsi pasitelkiant 3D rekonstrukcijos vaizdus gyvuosiuose organizmuose, nėra žinomas (Paper I). Dauguma taikytų vizualizavimo

metodų reikalavo gyvūnų žūties (Chen *et al*. 2011; Brun *et al*. 2014; Lee, An 2014; Böhme *et al*. 2017; Paper I).

Iki šiol KT poveikio mechanizmai organizmui nėra gerai ištirti, todėl jie galėtų būti aiškinami remiantis kitų ND sukeltų efektų pavyzdžiu (Murugan *et al*. 2015). Dauguma tyrimų aiškino KT toksiškumą, susijusį su jų skilimo produktais (metalais) (Ipe *et al*. 2005; Ribeiro *et al*. 2012; Katsumiti *et al*. 2014; Rocha *et al*. 2015; Santana *et al*. 2015), tačiau chemiškai stabilių KT poveikio mechanizmai vandens organizmams liko neišaiškinti. Manoma, jog KT poveikis žuvims ankstyvajame jų vystymesi yra susijęs su oksidaciniu stresu (Blickley *et al*. 2014). Nustatyta, kad KT ir jų skilimo produktai generuoja ROS, ir jų sukeltas oksidacinis stresas sutrikdo pro- ir antioksidantų pusiausvyrą organizme (Basha, Rani 2003; Blickley *et al*. 2014). Gao (2016) tyrė galimą AgND toksiškumo mechanizmą. Jo nuomone, ant žuvų embrionų choriono kaupiasi ND, todėl yra sutrikdomas deguonies patekimas pro chorioną į embriono vidų ir dėl sukeltos hipoksijos registruojamas ŠD sulėtėjimas (bradikardija) (Gao 2016).

Tyrimais yra įrodyta, kad hipoksiją vandens organizmams gali sukelti gamtoje esančios labai smulkios organinės (dėl eutrofikacijos vandens telkiniuose susidaro didelis kiekis melsvabakterių (HAB)) arba neorganinės (molis) kilmės nanomedžiagos (Grieg *et al*. 2005; Julien, Bergeron 2006; Wyatt 2009). Taip pat kaip ir ND šios nano- ir mikrodydžio medžiagos gali mechaniškai užkimšti žuvų embrionų choriono poras ir neigiamai paveikti besivystantį organizmą (Grieg *et al*. 2005; Julien, Bergeron 2006; Louhi *et al*. 2008; Wyatt 2009). Todėl būtina ištirti ir palyginti nanoskalės ribose esančių dirbtinai sukurtų (KT) ir gamtoje egzistuojančių (HAB, molis) medžiagų toksikologinį potencialą vandens organizmams. Ilgalaikio KT poveikio tyrimai ir jų palyginimas su kitomis organinės ir neorganinės kilmės medžiagomis yra svarbūs tiek toksikologiniu, tiek aplinkosauginiu požiūriu ir turi būti tęstini, siekiant geriau suprasti jų poveikio mechanizmą ne tik žuvims, bet ir kitiems vandens organizmams skirtingais jų vystymosi etapais bei žmogui.

Darbo naujumas:

1. Pirmą kartą karboksilintų CdSe/ZnS KT ir Cd atskirai toksikologinis potencialas buvo kompleksiškai įvertintas trumpalaikių ir ilgalaikių tyrimų metu, naudojant vaivorykštinį upėtakį ankstyvosiose jo vystymosi stadijose.
2. Nustatyta, kad tirti CdSe/ZnS KT inkubaciniame vandenyje buvo chemiškai stabilūs, nes metalai neatsipalaidavo iš KT struktūros ir nesukėlė MT indukcijos test-organizmuose.

3. Pirmą kartą nustatyta, kad inkubaciniame vandenyje tirti CdSe/ZnS KT sudarė agregatus ir aglomeravosi ant embrionų choriono ir lervų paviršiaus (žiaunų srityje).
4. Pirmą kartą parodyta, kad tirti CdSe/ZnS KT nepateko į embrionų vidų, bet užstrigo chorione ir užkimšo choriono poras, tuo pažeisdami jo vientisumą.
5. Konfokalinės fluorescencinės mikroskopijos, spektroskopijos ir histologijos taikymo galimybės buvo praplėstos ir panaudotos, tiriant KT kaupimąsi, patekimą ir pasiskirstymą gyvuose ir negyvuose žuvų embrionuose bei lervose.
6. Eksperimentų su Cd ir gamtoje esančiomis organinėmis ir neorganinėmis nano- ir mikrodydžio medžiagomis rezultatai parodė, kad chemiškai stabilių KT poveikis žuvims ankstyvajame jų vystymesi yra mechaninio pobūdžio.

Mokslinė darbo reikšmė:

1. Ištirtas ilgalaikis KT ir Cd poveikis žuvims ankstyvosiose jų vystymosi stadijose, naudojant kompleksą metodų, yra svarbus žingsnis siekiant išaiškinti metalų pagrindu sukurtų ND poveikio mechanizmus.
2. Gauti rezultatai suteikia naujų žinių apie KT embriotoksiškumą ir nanotoksiškumą vandens organizmams.
3. Nustatytas tirtų KT kaupimasis ir pasiskirstymas žuvų embrionuose suteikia naujų žinių apie choriono struktūrą, jo funkcijas ir padeda suprasti ND prasiskverbimo pro biologinius barjerus mechanizmus kituose organizmuose ir žmoguje.
4. KT, gyvų žuvų embrionų ir lervų vizualizavimas, naudojant konfokalinę fluorescencinę mikroskopiją, atveria naujų galimybių ne tik KT, bet ir kitų ND kaupimuisi ir pasiskirstymui organizmuose nustatyti.
5. Rezultatai suteikia naujų žinių kaip KT fizikocheminės savybės siejasi su jų poveikiu organizmui.
6. Eksperimentų su žuvimis ir gamtoje esančiomis nano- ir mikrodydžio medžiagomis duomenys leidžia manyti, kad chemiškai stabilių KT agregatai veikia test-organizmus mechaniniu būdu, o tai gali būti naudojama, aiškinant ne tik KT, bet ir kitų ND agregatų galimus poveikio mechanizmus vandens organizmams.
7. Gauti duomenys gali būti naudingi sparčiai vystantis nanotechnologijų ir nanotoksikologijos sritims.

Praktinė darbo reikšmė:

1. Gauti tyrimo rezultatai padės spręsti KT ir metalų ekotoksiškumo problemas.
2. Eksperimentinių tyrimų duomenys sudaro prielaidas gamtinių vandenų testavimui, KT ir kitų ND poveikio pasekmių vandens organizmams prognozavimui.
3. Gauti duomenys yra naudingi, vertinant vandens ekotoksikologinę būklę, ir prisidės prie nuotekų toksinio poveikio kompleksinės vertinimo sistemos tobulinimo Lietuvoje.
4. Kompleksinio tyrimo metu gautos naujos žinios prisidės kuriant aplinkai saugias ND.
5. Šio tyrimo duomenys bus naudingi ND reglamentavimui, reguliavimui ir standartizavimui.

Darbo tikslas:

Ištirti karboksilintų CdSe/ZnS KT ir Cd toksikologinį potencialą, siekiant nustatyti KT stabilumą, jų kaupimąsi, patekimą, pasiskirstymą ir išaiškinti KT poveikio mechanizmus žuvyse ankstyvosiose jų vystymosi stadijose.

Darbo uždaviniai:

1. Ištirti ir palyginti KT ir Cd poveikį vaivorykštinio upėtakio embrionams ir lervoms, vykdant trumpalaikius ir ilgalaikius eksperimentus.
2. Ištirti KT cheminį stabilumą ir jų elgesį inkubaciniame tirpale.
3. Nustatyti sąveiką tarp KT ir žuvų embrionų paviršių; KT patekimo galimybes per chorioną.
4. Nustatyti KT kaupimąsi ir pasiskirstymą vaivorykštinių upėtakių, dryžuotojų danijų ir perlinių guramių embrionuose ir lervose.
5. Įvertinti gautus duomenis ir išaiškinti KT poveikio mechanizmus žuvyse ankstyvajame jų vystymesi.

Ginamieji teiginiai:

1. Karboksilinti CdSe/ZnS KT ir Cd sukelia neigiamą poveikį vaivorykštinio upėtakio embrionams ir lervoms, vykdant trumpalaikius ir ilgalaikius eksperimentus.
2. KT yra chemiškai stabilūs ir metalai neatsipalaiduoja iš jų struktūros.
3. KT yra linkę agreguotis tirpale ir aglomeruotis test-organizmų paviršiuje.
4. KT neprasiskverbia pro žuvų embrionų chorioną.

5. KT kaupiasi ir pasiskirsto žuvų embrionų chorione ir lervų išoriniuose kūno audiniuose.
6. KT užkemša embrionų choriono poras ir pažeidžia jo vientisumą.
7. KT poveikis test-organizmams yra susijęs su KT mechaniniu poveikiu.

TYRIMŲ MEDŽIAGA IR METODIKA

Svarbiausi šioje disertacijoje naudojami metodai yra pateikti 1 paveiksle. Išsamesnė informacija apie naudotus metodus pateikta kiekvienoje publikacijoje (I–XI).

Puslaidininkiniai kvantiniai taškai (KT) (CdSe/ZnS-COOH kat., Nr. A10200) buvo įsigyti iš „Life Technologies“ (JAV) (2 pav.). Šie KT turi stiprią fotoliuminescenciją (FL) raudoname spektro regione, kurio FL maksimumas yra ties 625 nm. KT pradinis tirpalas buvo 100 µL 8 µM KT, kuris buvo ištirpintas gręžinio vandenyje, kad galutinė KT koncentracija inkubaciniame vandenyje būtų 4 nM.

Cd tirpalai (0,5; 1,0; 2,0; 4,0 ir 8,0 µg/L) paruošti, naudojant cheminei analizei skirtą Cd druską ($CdCl_2 \cdot H_2O$) („Reachim“, Rusija). Pradinis (koncentruotas) tirpalas buvo ruošiamas, ištirpinant reikiamą Cd druskos kiekį distiliuotame vandenyje, o galutinė koncentracija inkubaciniame vandenyje perskaičiuojama pagal metalo jonų kiekį.

Siekiant išaiškinti galimą KT mechaninį poveikį test-organizmams (1 lentelė), buvo vykdyti eksperimentai su gamtoje esančiomis nano- ir mikrodydžio organinės ir neorganinės kilmės medžiagomis (3 pav.): homogenizuota melsvabakterių biomase (HAB) (0; 12,5; 25; 50; 100 ir 200 mg ss/L) ir moliu (0; 0,375; 0,750; 1,5; 3,0; 6,0 ir 12,0 g/L). Nustatyta, kad eksperimentų pradžioje KT dydis buvo 50–100 nm, HAB sudarančių dalelių – 60–700 nm, o molio – 90–1920 nm.

Naudoti toksikologiniai metodai išsamiai aprašyti I–XI publikacijose. KT kaupimuisi, patekimui ir pasiskirstymui žuvyse nustatyti naudoti fizikiniai metodai pateikti I, II ir III publikacijose. Cheminiai Cd akumuliacijos nustatymo metodai naudojant atominės absorbcijos spektrofotometriją aprašyti I, III, V ir X publikacijose. Metalotioneino (MT) kiekio matavimo biocheminis metodas pateikiamas X publikacijoje. Tyrimuose naudotų cheminių elementų koncentracijų ir fizikocheminių parametrų vandenyje matavimo metodai aprašyti atitinkamose publikacijose (I–III, V–VI, VIII ir X).

Tyrimų metu taikyti statistiniai metodai yra išsamiai aprašyti kiekvienoje publikacijoje (I–XI).

Eksperimentai su test-organizmais buvo vykdomi vienodomis kontroliuojamomis sąlygomis, po tris pakartojimus ir po 20 ar 30 individų kiekviename pakartojime. Tyrimai atlikti su skirtingomis žuvų rūšimis:

vaivorykštiniu upėtakiu, dryžuotąja danija, perliniu guramiu. Eksperimentų metu buvo vertinamas test-medžiagų poveikis vaivorykštinio upėtakio biologiniams parametrams (1 lentelė).

REZULTATAI IR JŲ APTARIMAS

KT ir Cd poveikis embrionams

CdSe/ZnS-COOH KT ir Cd toksikologinis potencialas buvo ištirtas vaivorykštinio upėtakio embrionams. Eksperimentinio tyrimo metu, kuris tęsėsi 12 parų, stebėtas embrionų mirtingumas ir ŠD (2 lentelė; 4 pav.; Paper VI: Fig. 2; Paper VII: Table 2). KT neturėjo reikšmingo poveikio embrionų mirtingumui (2 lentelė Paper VI: Fig. 2A). Tuo tarpu embrionų mirtingumas veikiant juos Cd, reikšmingai skyrėsi nuo embrionų mirtingumo kontrolinėje grupėje po 1, 4, 8 ir 12 parų (2 lentelė; Paper VII: Table 2). Atlikus lyginamąją KT ir Cd poveikių vaivorykštinio upėtakio embrionams mirtingumo analizę, nustatyta, kad KT poveikis reikšmingai skiriasi nuo Cd poveikio po 8 ir 12 parų (2 lentelė). Zhang *et al*. (2012a; 2012b) nustatė, kad CdSe-MPA, CdTe-TGA, CdTe-TGA KT dryžuotosios danijos lervoms 120 val. LC50 reikšmės yra 1,98 mg/L, 185,9 nM ir 22,31 mg/L, atitinkamai. Hardman (2006) teigė, kad KT toksiškumą dažniausiai lemia metalai (Cd, Te, Zn ir kt.), esantys jų šerdyje. Oksidacijos ar fotolizės reakcijų metu metalai gali atsipalaiduoti iš KT ir sukelti didelį organizmų mirtingumą, tačiau stabilūs KT neturi reikšmingo toksinio poveikio (Hardman, 2006). Eaton *et al*. (1978), Levit (2010), Zhang *et al*. (2012a), Calfee *et al*. (2014) duomenys parodė reikšmingą žuvų embrionų ir lervų mirtingumo padidėjimą veikiant juos labai mažomis Cd koncentracijomis.

Nustatytas reikšmingas KT poveikis embrionų ŠD, kuris buvo mažesnis nei embrionų kontrolėje (4 pav.; Paper VI: Fig. 2B). Embrionų ŠD, pakitimai buvo susiję su KT poveikio trukme (4 pav.; Paper VI: Fig. 2B). Embrionų ŠD veikiant KT ir Cd, po 1 ir 4 parų reikšmingai skyrėsi nuo poveikio po 12 parų (4 pav.; Paper VI: Fig. 2B; Paper VII: Table 2). Nenustatytas reikšmingas skirtumas tarp KT ir Cd poveikių embrionų ŠD viso eksperimento metu (4 pav.). KT paveiktų embrionų ŠD po 8 ir 12 parų nesiskyrė nuo kontrolės, kas rodo embrionų ŠD atsistatymą. Tačiau literatūroje yra šiek tiek duomenų, aiškinančių atsistatymo mechanizmus, veikiant žuvis metalais ankstyvajame jų vystymesi (Witeska *et al*. 2014).

KT poveikis lervoms

Trumpalaikis KT ir Cd poveikis buvo nustatytas vaivorykštinio upėtakio lervų mirtingumui, ŽVD, ŠD ir elgsenos reakcijai, priklausomai nuo

paveiktos vystymosi stadijos (paveikti embrionai para iki ritimosi – pirmasis bandymas, paveiktos vienadienės lervos – antrasis bandymas), cheminės medžiagos tipo (KT ir Cd) ir poveikio trukmės (24 ir 96 val.) (Paper II: Fig. 1). Pirmojo bandymo metu nustatyta, kad KT neturėjo reikšmingo poveikio lervų mirtingumui ir ŽVD, palyginti su kontrole (Paper II: Fig. 1A and B). Antrojo bandymo pabaigoje KT sukėlė reikšmingą lervų mirtingumo ir ŽVD padidėjimą, palyginti su kontrole. Abiejų bandymų pabaigoje, t.y. po 96 val. poveikio Cd, lervų mirtingumas reikšmingai padidėjo, o lervų ŽVD sumažėjo, palyginti su kontrole (Paper II: Fig. 1A and B).

Pirmojo bandymo metu KT nesukėlė reikšmingo lervų ŠD sumažėjimo, o Cd sukėlė ŠD reikšmingą sumažėjimą. Antrojo bandymo metu buvo nustatytas reikšmingas lervų ŠD sumažėjimas paveikus jas tiek KT tiek Cd (Paper III: Fig. 1C). Abiejuose bandymuose nustatyta, kad KT ir Cd sukėlė reikšmingus elgsenos pokyčius po 24 ir 96 val., palyginti su kontrole (Paper II: Fig. 1D).

Tyrimų rezultatai parodė, jog lervų ŽVD priklausė nuo cheminės medžiagos tipo ir paveiktos organizmo vystymosi stadijos (Paper II: Fig. 1B). Nustatyta, kad KT ir Cd poveikis vaivorykštinio upėtakio lervų biologiniams parametrams (mirtingumui, ŽVD, ŠD ir elgsenos reakcijai) daugeliu atvejų skyrėsi: mirtingumas nuo KT reikšmingai skyrėsi nuo Cd poveikio abiejų bandymų pabaigoje, ŽVD – pirmojo bandymo pabaigoje ir antrojo bandymo pradžioje, ŠD – abiejų bandymų pabaigoje ir elgsenos reakcija – antrojo bandymo pabaigoje (Paper II: Fig. 1).

Nustatyta, kad lervų ŽVD ir ŠD pirmojo bandymo metu po 96 val. poveikio KT priklausė nuo paveiktos organizmo vystymosi stadijos, o antrojo bandymo metu KT paveiktų lervų ŽVD ir ŠD priklausė nuo poveikio trukmės (Paper II: Fig. 1B and C). Taip pat lervų mirtingumas ir ŽVD po poveikio Cd reikšmingai skyrėsi ir priklausė nuo poveikio trukmės ir paveiktos organizmo vystymosi stadijos. Šie duomenys parodė, kad Cd paveiktos lervos buvo jautresnės nei paveikti embrionai (Paper II: Fig. 1).

Ilgalaikio tyrimo (14 parų) rezultatai parodė, kad KT sukėlė reikšmingą mirtingumo, ŽVD ir elgsenos reakcijos padidėjimą, palyginti su kontrole (Paper III: Table 3). Tačiau po 1 ir 4 parų poveikio lervų mirtingumas ir ŽVD reikšmingai nesiskyrė nuo kontrolės. Taip pat KT nesukėlė reikšmingo lervų ŠD padidėjimo viso tyrimo metu (Paper III: Table 3). Tačiau KT sukėlė reikšmingą lervų SKMP sumažėjimą po 14 parų poveikio, palyginti su kontrole (Paper III: Table 3). Panašūs rezultatai buvo gauti ir kitų autorių tyrimuose, kuriuose KT sukėlė neigiamą poveikį dryžuotosios danijos lervų mirtingumui, kvėpavimo ir elgsenos pokyčiams (King-Heiden *et al*. 2009;

Duan *et al*. 2013; Zolotarev *et al*. 2013). Duan *et al*. (2013) padarė išvadą, kad dryžuotojų danijų ŠD priklausė nuo KT koncentracijos ir poveikio trukmės. Ilgėjant poveikio laikui, ŠD vis labiau lėtėjo, palyginti su kontroline grupe.

KT stabilumas

Tyrimai parodė, jog inkubaciniame tirpale KT sudaro agregatus, o ant test-organizmų paviršiaus – aglomeratus (Paper I: Fig. 1), sudarydami didesnes, už nanoskalės ribų esančias, struktūras. Todėl, pirmiausia, aiškinantis KT poveikio mechanizmą turi būti atsižvelgta į KT fizikocheminių savybių pokyčius (Hardman 2006). Vaivorykštinio upėtakio embrionų inkubacinis vanduo eksperimento pradžioje buvo skaidrus ir homogeniškas (Paper I: Fig. 1A and C). Tačiau po 10 parų poveikio KT buvo pastebėtos didelės oranžinės / rudos spalvos nehomogeniškos nuosėdos, esančios inkubaciniame vandenyje ir ant embrionų (Paper I: Fig. 1B and D). Inkubaciniame vandenyje KT sudarė plika akimi matomus agregatus, kurie, apšvietus UV šviesa, tapdavo raudoni.

KT spektrai buvo palyginti eksperimento pradžioje ir po 10 parų poveikio (Paper I: Fig. 1E). Sunormavus FL spektrus, buvo nustatyta, jog inkubacinis vanduo paveikė KT charakteristikas. Eksperimento pradžioje FL spektro juostos plotis buvo siauresnis negu eksperimento pabaigoje. Taip pat KT fotoliuminescencijos intensyvumas inkubaciniame vandenyje sumažėjo per 10 parų. Inkubacijos laikotarpiu buvo aptiktas FL spektrų maksimumo poslinkis nuo 625 nm iki 628 nm. FL intensyvumo sumažėjimas, ilgėjant inkubacijos trukmei, buvo susijęs su KT agregavimu inkubacijos vandenyje (Kulvietis *et al*. 2011). KT turi neigiamą krūvį, todėl inkubaciniame vandenyje sąveikavo su ten esančiais jonais (Paper I: Fig. 1). KT agregavimas yra pastebėtas Gagné *et al*. (2008), King-Heiden *et al*. (2009), Zolotarev *et al*. (2012).

KT stabilumo analizė leido daryti prielaidą, jog ND toksiškumo mechanizmas negali būti aiškinamas vien tik metalų poveikiu, kuris gali atsirasti joms subyrėjus. Aiškinantis KT poveikio mechanizmą, naudingą informaciją suteikė metalų kiekio įvertinimas tiek test-organizmuose, tiek jų inkubaciniame vandenyje. Metalų įvertinimas leido kiekybiškai nustatyti KT kiekį, nes į šią analizę yra įtraukiamas visas pasirinkto metalo kiekis, esantis KT struktūroje (Zarco-Fernández *et al*. 2016). Maksimali Cd koncentracija buvo nustatyta vaivorykštinio upėtakio embrionuose, kurie buvo paveikti KT vieną parą (Paper I: Fig. 1F). Tačiau po keturių parų poveikio KT Cd

koncentracija sumažėjo. Be to, buvo įvertinta Cd koncentracija viršutiniuose ir apatiniuose inkubacinio vandens sluoksniuose eksperimento pabaigoje (Paper I: Fig. 1G). Nustatyta, kad didžioji dalis Cd yra koncentruota nuosėdose.

Įvertinus MT kiekį vaivorykštinio upėtakio lervose buvo nustatyta, jog KT (4nM) nesukėlė reikšmingo MT kiekio padidėjimo, palyginti su kontrole (Paper X: Fig. 4). Tai leidžia daryti prielaidą, kad KT nesubyrėjo ir metalai neatsipalaidavo iš jų struktūros. Šie rezultatai sutampa su Fischer et al. (2006) duomenimis, kurie rodo, kad KT dangalas apsaugo juos nuo subyrėjimo.

Šiame tyrime buvo naudojama konfokalinė fluorescencijos mikroskopija, skirta vizualizuoti KT kaupimąsi, patekimą ir pasiskirstymą skirtingų žuvų rūšių audiniuose: vaivorykštinio upėtakio, dryžuotosios danijos ir perlinio guramio embrionuose (Paper I: Fig. 2). Vaivorykštinio upėtakio embrionai yra santykinai dideli objektai konfokaliniam vaizdinimui atlikti. Be to, embriono audiniai beveik neskaidrūs. Todėl buvo vaizdinama tik nedidelė embrionų dalis (Paper I: Fig. 2B). Papildomas tyrimas buvo atliktas su kitomis žuvų rūšimis: perlinio guramio ir dryžuotosios danijos embrionais, kad būtų galima vizualizuoti KT pasiskirstymą visame embriono tūryje (Paper I: Fig. 2A and C).

3D rekonstrukcija buvo atlikta iš 2D vaizdų, kurie buvo gauti iš įvairių optinių pjūvių (Paper I: Fig. 2). Priklausomai nuo užsibrėžto tikslo buvo naudojama iki 200 vnt. 2D vaizdų, kurių žingsnis (Δz) buvo 4–13,3 μm. Iš pradžių kiekvienas 2D vaizdas buvo sudarytas iš šviesos pralaidumo, autofluorescencijos ir KT fotoliuminescencijos signalų. Tačiau šviesos pralaidumo signalui gauti trūko tikslumo, reikalingo 3D vaizdavimui atlikti. Todėl 3D rekonstrukcija buvo pagrįsta tik autofluorescencija ir KT FL signalais. Atlikus tyrimus paaiškėjo, kad KT žuvų embrionuose pasiskirsto nevienodai (Paper I: Fig. 2). Remiantis 2D vaizdais, tik embrionų išorėje (baltos spalvos apskritimai) buvo aptikta KT fotoliuminescencija (Paper I: Fig. 2B). Taigi, nepriklausomai nuo žuvų rūšies, KT nepatenka į jų embrionų vidų.

Palyginamoji 3D rekonstrukcijos vaizdų dryžuotosios danijos ir vaivorykštinio upėtakio embrionų analizė parodė, kad KT yra įsiterpę į jų chorionus (Paper I: Fig. 3). KT fotoliuminescencija užregistruota viduje autofluorescencinio 3D vaizdo abiejų tiriamų rūšių embrionuose (Paper I: Fig. 3). Taigi, KT kaupimasis ir pasiskirstymas chorione buvo patvirtintas keliais būdais: atlikus 2D embrionų vaizdinimą konfokaliniu mikroskopu, iš

3D rekonstrukcijos vaizdų ir ištyrus žuvų chorioną po lervų išsiritimo (Paper I: Fig. 2 and 3).

Histologinių pjūvių analizė leido patvirtinti išvadą, kad KT yra tik embrionų chorione (Paper I: Fig. 6). Žuvų embrionų trynio maišelyje, perivitelinėje ertmėje KT fotoliuminescencija nebuvo užregistruota (Paper I: Fig. 6). Taigi, histologinių pjūvių spektrinė analizė suteikė naujų žinių apie embrionų morfologiją, kuri leido tiksliai nustatyti KT buvimo vietą embrione, šiuo atveju tik ant choriono (Paper I: Fig. 6).

KT kaupimasis ir pasiskirstymas žuvų lervose

Gauti rezultatai parodė, kad Cd koncentracija lervose buvo statistiškai patikimai aukštesnė, palyginti su kontrole ir nepriklausė nuo poveikio trukmės (Paper X: Fig. 3). Dvimačiuose šviesaus lauko ir fluorescenciniuose vaizduose KT pasiskirstymas apima vieną lervos kūno sritį: KT fotoliuminescencija buvo užregistruota tik lervų žiaunų srityje po 24 val. poveikio KT (4 nM) (Paper III: Fig. 1C).

KT poveikio žuvims ankstyvajame jų vystymesi mechanizmai

KT poveikio mechanizmams išsiaiškinti buvo panaudota konfokalinė mikroskopija ir gautų vaizdų 3D rekonstrukcija (Paper I: Fig. 4). Konfokalinė mikroskopija leido užtikrinti realų KT pasiskirstymą test-organizme, nes žuvų embrionai vaizdinimui atlikti buvo gyvi. Rocha *et al*. (2017) ir Hardman (2006) teigė, kad KT poveikio mechanizmai priklauso nuo jų patekimo kelio į organizmus. Kadangi KT suformuoja agregatus (Paper I: Fig. 1), tai didesnėms struktūroms patekti į test-organizmus tampa sudėtingiau (Hardman 2006; Cheng *et al*. 2007). Pirmiausia, KT apkimba žuvų embrionų chorioną (Paper I: Fig. 4B), kuris yra barjeras cheminėms medžiagoms patekti į jų vidų (Ninness *et al*. 2006; Jaramillo *et al*. 2015). Vėliau vystantis embrionui, chorionas plonėja ir plyšta (Jaramillo *et al*. 2015), tokiu būdu KT gali patekti į embrioną (Paper I: Fig. 4). Tik išsiritus žuvų lervoms, KT agregatai apkimba visą lervos kūną, ypač žiaunas (Paper III: Fig. 1C). Žiaunos yra sritis, kurioje buvo fiksuojamas didžiausias KT fotoliuminescencijos intensyvumas (Paper III: Fig. 1C) ir stebimas didžiausias gleivių susidarymas. Tyrimo rezultatai rodo, kad KT sukėlė lervų ŽVD pokyčius (Paper II: Fig. 1B; Paper III: Table 2). Tyrimai, atlikti su kitomis ND rūšimis, patvirtino gautus rezultatus, kad ND poveikio metu sukelia gleivių išsiskyrimą vaivorykštinio upėtakio ir dryžuotosios danijos lervose, todėl pasireiškia žiaunų hiperplazija (Federici *et al*. 2007; Smith *et*

al. 2007; Mansouri, Johari 2016). Kadangi lervos dar nesimaitina iš išorės (endogeninė mityba), todėl patekti KT per virškinamąjį traktą nėra galimybės (Lei *et al*. 2011; Duan *et al*. 2013). Lei *et al*. (2011) pažymėjo, kad KT negali patekti į lervas ir per trynio maišelį dėl jame esančio didelio lipidų kiekio.

Nustatyta, kad KT formuoja įvairių dydžių agregatus ant choriono vaivorykštinio upėtakio embrionų po 1–2 parų poveikio KT (Paper I: Fig. 4B). Be to, buvo nustatyta, kad KT agregatai padengiami choriono gleivėmis po 3–4 parų poveikio KT (Paper I: Fig. 4C). KT agregatai sąveikauja su choriono gleivėmis (Paper I: Fig. 4C). Taip pat ilgėjant inkubacijos trukmei, aptikta choriono išorinio sluoksnio pokyčių (Paper I: Fig. 4D). KT įsiterpia į embrionų chorioną ir sukelia choriono paviršiaus pažeidimų (Paper I: Fig. 4D). Po 5–6 parų inkubacijos KT agregatų ir gleivių kompleksas atsiskiria nuo embriono (Paper I: Fig. 4D). KT po 8–12 parų poveikio pažeidžia vaivorykštinio upėtakio embrionų išorinės membranos vientisumą (Paper I: Fig. 4E).

Kad embrionas išskiria gleives patvirtina ir Songe *et al*. (2016). Galima teigti, kad dėl išsiskyrusių gleivių ant choriono, pirmieji KT iš vandens prikimba prie choriono, taip sudarydami su kitais KT kondensacijos centrus ir šie dideli aglomeratai kaupiasi ant embriono paviršiaus ir jų sankaupos didėja. Kadangi vėliau dalis jų atsiskiria nuo choriono paviršiaus (Paper I: Fig. 4), todėl dalis KT ir baltyminių struktūrų kompleksų plaukioja vandens tirpale (Paper I: Fig. 1B and D). Kita vertus, į vandenį nuo embrionų paviršiaus atsiskiria biomolekulės, kurios yra KT kondensacijos centrais jau pačiame inkubaciniame vandenyje, kurie palaipsniui sedimentuoja ant dugno ir embrionų paviršiaus.

Atlikus detalesnį embrionų vaizdinimą, paaiškėjo KT pasiskirstymo chorione mechanizmas (Conference abstract V: Fig. 1D). KT kaupiasi chorione ir užkemša jo poras (Conference abstract V: Fig. 1). KT fotoliuminescencija matoma susitelkusi išoriniame choriono sluoksnyje, sudarytame iš cilindro formos savitosios fluorescencijos darinių (Conference abstract V: Fig. 1D). Konfokalinės mikroskopijos ir histologijos pjūvių analizės būdu nustatyta, jog vaivorykštinio upėtakio chorioną sudaro trys sluoksniai, o KT kaupiasi ant embrionų choriono paviršiaus ir pasiskirsto pirmame choriono sluoksnyje (neskelbti duomenys). Parodyta, kad erdvinis KT fotoliuminescencijos išsidėstymas chorione priklauso nuo KT agregatų dydžio (Conference abstract V: Fig. 1D). Mažų KT agregatų fotoliuminescencija yra susitelkusi išorinio choriono sluoksnio cilindrinės formos savitosios fluorescencijos darinių užimamoje erdvėje (Conference

abstract V: Fig. 1). Esant didesniems agregatams, KT fotoliuminescencijos pasiskirstymas užima erdvinę sritį, apimančią daugiau nei vieną poros užimamą erdvę (Conference abstract V: Fig. 1). Per poras vyksta medžiagų ir dujų apykaita į embrioną ir iš jo (Jaramillo *et al*. 2015). Nustatyta, kad 3D rekonstrukcijoje esančių porų dydis siekia 1,1±0,2 μm, atstumas tarp porų (nuo centro iki centro) 2,2±0,3 μm (Conference abstract V: Fig. 1). Dryžuotosios danijos choriono porų dydis siekia 0,5–0,7 μm, o atstumas tarp porų centrų 1,5–2,0 μm (Rawson *et al*. 2000). Dėl šios priežasties, nano- ir mikrodydžio medžiagos gali prasiskverbti pro choriono poras (Cheng *et al*. 2007).

KT agregatai gali destruktyviai veikti choriono vientisumą (Paper I: Fig. 4). Duomenų apie KT kaupimąsi, patekimą ir pasiskirstymą vaivorykštinio upėtakio embrionose ir lervose nėra. Daugiausia duomenų yra aptinkama apie kitų ND kaupimąsi, patekimą ir pasiskirstymą dryžuotosios danijos embrionuose ir lervose (Ag ND – Asharani *et al*. (2008); SiO_2 ND – Fent *et al*. (2010); Co_3O_3, CuO, NiO, ZnO ND – Lin *et al*. (2011); Al_2O_3, Ag ir Au NPs – Böhme *et al*. (2017); ZnO ND – Brun *et al*. (2014); Cu_2O, $CuCl_2$ ND – Chen *et al*. (2011); CdSe/ZnS KT – Lee, An (2014)).

Nors KT patekimo ir pasiskirstymo vaivorykštinio upėtakio embrionų viduje nenustatyta, tačiau tyrimo metu buvo aptikta keletas pažeistų embrionų. KT sukėlė embrionų stuburo iškrypimą, kraujo išsiliejimą, ritimąsi galva (Paper I: Fig. 7).

Vystymosi sutrikimų buvo nustatyta ir vaivorykštinio upėtakio lervose, paveikus jas KT (Paper III: Fig. 2). Matomos tokios pažaidos kaip kraujo išsiliejimai galvos srityje ir trynio maišelyje (Paper III: Fig. 2A and B). Atsiradusios embrionų pažaidos yra sietinos su embrionų ir lervų fiziologinių funkcijų pokyčiais, kurie gali būti sąlygoti deguonies trūkumo (hipoksijos), medžiagų apykaitos sutrikdymo (Shang, Wu 2004; Shang *et al*. 2006; Gao 2016).

Papildomai buvo vykdyti eksperimentai su melsvabakterių biomase (HAB) ir molio dalelėmis, kad būtų įsitikinta, jog KT poveikio mechanizmai test-organizmams yra susiję su jų mechaniniu poveikiu (porų užkimšimo ir deguonies apykaitos sutrikdymu). Atlikta HAB analizė parodė, kad joje yra mažas kiekis toksinų, galinčių pakenkti vandens organizmams (Paper IV: Table 1). Kaip ir kitos gamtoje esančios smulkios dalelės, HAB ir molis sutrikdo dujų ir medžiagų apykaitą pro chorioną, prikimba prie suaugusių žuvų ir lervų žiaunų ir apsunkina jų kvėpavimą (Lapointe *et al*. 2004; Shang, Wu 2004; Grieg *et al*. 2005; Julien, Bergeron 2006; Shang *et al*. 2006; Gao 2016). Eksperimentų metu buvo nustatyta, kad HAB, molio ir KT poveikis

yra panašus (Paper I, IV, V). Duomenys parodė, kad vaivorykštinio upėtakio jauniklių didžiausias mirtingumas (100 proc.) buvo, esant didžiausiai HAB koncentracijai (200 mg ss/L) (Paper IV: Fig. 4B). Mažesnėse HAB koncentracijose vaivorykštinio upėtakio jauniklių mirtingumas buvo nedidelis arba jo nebuvo visai (Paper IV: Fig. 4B). Paveikus vaivorykštinio upėtakio embrionus tomis pačiomis HAB koncentracijomis, po 96 val. poveikio nebuvo nustatyta reikšmingo embrionų mirtingumo padidėjimo, tačiau buvo nustatytas ŠD reikšmingas sumažėjimas po 1 ir 4 parų poveikio (duomenys neskelbti).

Rezultatai parodė, kad, didėjant molio koncentracijai ir ilgėjant poveikio trukmei, embrionų mirtingumas reikšmingai nepadidėjo, palyginti su kontrole. Tačiau po 4 ir 8 parų poveikio pastebėtas nedidelis embrionų mirtingumo padidėjimas. Taip pat rezultatai parodė, kad molis po 1 ir 4 parų poveikio sukėlė embrionų ŠD sulėtėjimą (bradikardiją), bet po 8 parų poveikio ŠD panašiai kaip ir KT atveju buvo kontrolės ribose (Paper I: Fig. 9).

Visų vykdytų eksperimentų metu buvo pastebėta suintensyvėjusi test-organizmų gleivių ekskrecija. Tai rodo, kad gamtoje esančios nano- ir mikrodydžio medžiagos ir dirbtinai sukurti KT test-organzimus veikia neigiamai, sukeldami ne tik gleivių išsiskyrimą, bet ir kitus fiziologinius (ŠD ir ŽVD) pokyčius (duomenys neskelbti). Tačiau šie rezultatai reikalauja išsamesnių papildomų tyrimų, aiškinančių vandens organizmų atsakus į skirtingos kilmės nanomedžiagas.

Tyrimai parodė, kad KT, HAB ir molis turi panašų poveikį žuvims ankstyvose jų vystymosi stadijose. KT kaip ir gamtoje esančios smulkios organinės ir neorganinės kilmės medžiagos kaupiasi ant choriono, lervų žiaunose, todėl susidaro nepalankios organizmui vystymosi sąlygos. Šių medžiagų neigiamas poveikis organizmui gali būti siejamas su deguonies trūkumu. Yra žinoma, kad hipoksija sukelia žuvų mirtingumą, jų vystymosi ir reprodukcinius sutrikimus (Shang, Wu 2004; Shang *et al*. 2006; Gao 2016). Hipoksijos sukelti pokyčiai gali būti susiję su reaktyvių deguonies formų susidarymu ir oksidaciniu stresu (Shang, Wu 2004; Shang *et al*. 2006; Wu 2009) ir kitais pokyčiais, vykstančiais molekuliniame lygmenyje žuvyse ankstyvosiose jų vystymosi stadijose (Gao 2016).

Apibendrinus KT poveiko mechanizmų rezultatus (5 pav.), paaiškėjo, kad karboksilinti CdSe/ZnS KT sudaro agregatus inkubaciniame vandenyje ir aglomeratus ant test-organizmų paviršiaus. Eksperimentų metu nebuvo nustatytas metalų atsipalaidavimas iš KT struktūros. KT agregatai neprasiskverbė į vaivorykštinio upėtakio, dryžuotosios danijos ir perlinio

guramio embrionų perivitelinę ertmę. Todėl galima teigti, kad šių žuvų chorionas apsaugo embrionus nuo KT. Tačiau KT įsiterpia į embrionų chorioną ir po 10–12 parų poveikio jie mechaniškai pažeidžia jo vientisumą, užkemša poras. Choriono vientisumo pažeidimai galėjo sukelti kraujo išsiliejimų vaivorykštinio upėtakio embrionų širdies srityje, jų ritimąsi galva ir kitų lervų pažaidų. Tyrimų rezultatai leidžia daryti prielaidą, kad hipoksija, kurią mechaniškai sukelia chemiškai stabilūs KT ar gamtoje esančios nano- ir mikrodydžio medžiagos, gali būti viena iš priežasčių, lemiančių funkcinius pokyčius žuvyse, ypač ankstyvose jų vystymosi stadijose. Tai gali būti naudinga, aiškinant ir prognozuojant ne tik KT, bet ir kitų ND galimus poveikio mechanizmus vandens organizmams.

IŠVADOS

1. Ilgalaikiai ir trumpalaikiai eksperimentiniai tyrimai parodė, kad karboksilinti CdSe/ZnS kvantiniai taškai (KT) (4 nM) reikšmingai nepaveikė vaivorykštinių upėtakių embrionų mirtingumo, bet sąlygojo reikšmingą lervų mirtingumą. Embrionų ir lervų mirtingumas reikšmingai didėjo, ilgėjant Cd (2 µg/L) poveikio trukmei. Kiti biologiniai rodikliai (kvėpavimo, augimo, vystymosi, elgenos) taip pat dažniausiai reikšmingai priklausė nuo KT ir Cd poveikio trukmės.
2. KT ir Cd poveikis vaivorykštiniams upėtakiams ankstyvajame jų vystymesi priklausė nuo cheminių medžiagų tipo ir paveiktos žuvų vystymosi stadijos. Cd buvo toksiškesnis embrionams ir lervoms nei KT, o paveiktos lervos buvo jautresnės nei paveikti embrionai KT ir Cd poveikiui.
3. Chemiškai stabilūs KT (Cd neatsipalaidavo iš KT struktūros) sudarė agregatus inkubaciniame vandenyje ir aglomeratus ant žuvų embrionų ir lervų kūno paviršiaus. Nustatyta, kad KT kaupiasi embrionų chorione ir užkemša jo poras. Tuo tarpu lervose KT kaupiasi žiaunų sityje.
4. KT paskirstymo kompleksinė analizė vaivorykštinių upėtakių, dryžuotojų danijų ir perlinių guramių embrionuose parodė, kad KT nepatenka į jų vidų. Todėl galima teigti, kad šių žuvų chorionas yra apsauginis barjeras nuo KT patekimo į embrionus. Tačiau ilgalaikiai tyrimai parodė choriono vientisumo pažeidimus: KT įsiterpimą į choriono paviršių; porų užkimšimą; išorinio sluoksnio defragmentaciją.
5. Gauti duomenys parodė, kad KT poveikis embrionų ir lervų biologiniams rodikliams nebuvo susijęs su metalų, įeinančių į KT struktūrą, toksiškumu, nes KT buvo chemiškai stabilūs, bet buvo susijęs su kitomis KT fizikocheminėmis savybėmis (geba sudaryti agregatus ir aglomeratus).
6. Metalų, gamtoje esančių nano- ir mikrodydžio medžiagų, poveikio žuvims ankstyvosiose jų vystymosi stadijose tyrimų rezultatai, metalotioneinų kiekio įvertinimas lervų audiniuose leidžia daryti prielaidą, kad chemiškai stabilių KT poveikis test-organizmams yra mechaninio pobūdžio.

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NOTES

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